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Clara Paolucci

Role of Nitric Oxide in the Maturation Process of Human Dendritic Cells

Thesis submitted in partial fulfilment of the requirements of the
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DECLARATION

This Thesis has been composed by myself and has not been used in any previous application for a degree. Clara De Palma helped me with some of the proliferation experiments. All sources of information are acknowledged by means of references.

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2 ABSTRACT

Nitric oxide (NO) generated by phagocytes at inflammation sites contributes to regulate immune responses through both autocrine and paracrine actions on bystander cells. Among the latter are dendritic cells (DCs). Little is known about regulation of DC function by NO, especially in the human system. In this thesis I report about the role of NO in two specific aspects of human DC maturation: i.e. endocytosis and ability to induce T cell activation. At variance with rodents, human DCs express the inducible isoform of the NO synthase neither when immature, nor after various treatment which induce DC maturation, indicating that regulation of these cells may occur only through exogenous NO. Exposure of DCs to NO, released by either bystander phagocytes or NO donors, reversed the inhibition of endocytosis induced by TNF- α . The intracellular accumulation of ceramide induced by TNF- α was also inhibited by NO. In addition, NO was found to exert an inhibitory effect downstream of the TNF- α -triggered ceramide accumulation, since NO donors reversed the inhibition of endocytosis induced by the cell-permeant C₂-ceramide. I then studied the role of NO on DC ability of triggering T cell activation. DCs were exposed to the NO donor DETA-NO during their maturation process induced by treatment with TNF- α or lipopolysaccharide, or by CD40 activation. After exposure to DETA-NO, DCs exhibit a significantly increased ability to activate T lymphocytes stimulated by mycobacterial antigens, *Staphylococcus aureus* Cowen strain B, allo-antigens, or cross-linking of the CD3-T cell receptor complex. This effect persists after removal of DETA-NO and is due to enhanced release by DCs of soluble factors, in particular IL-12. All the effects described above were mimicked by the membrane-permeant cyclic GMP analogue, 8-Br cyclic GMP, and prevented by inhibition of the soluble guanylyl-cyclase. The cGMP dependent modulation of DC

function reported here is due to a synergism between NO and the various maturation stimuli since neither the changes in endocytic function nor the enhanced T cell activation and IL-12 release were observed after DC exposure to DETA-NO only. These results provide the first evidence that NO acts as a co-signalling molecule regulating human DC response to maturation stimuli.

3 INTRODUCTION

3.1 Dendritic cells are professional antigen presenting cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) possessing a unique ability to induce primary immune responses by capturing and transferring information from the outside world to the cells of the adaptive immune system. Dendritic cells are a heterogenous cell population, residing in most peripheral tissues, concentrated at sites of interface with the environment (skin and mucosae), where they represent 1%–2% of the total cell number (Banchereau et al., 1998; Banchereau et al., 2000). In peripheral tissues, DCs internalise self and nonself antigens, which are then processed into proteolytic peptides and loaded onto major histocompatibility complex (MHC) class I and II molecules to be presented to T cells. Such presentation process is quite inefficient in peripheral DCs. However specific signals induce them entering into a developmental programme, called maturation, which transforms them into efficient APCs and T cell activators. Among these signals are bacterial and viral products, often referred to as danger signals, as well as inflammatory cytokines and other self-molecules, which induce maturation through direct interaction with specific receptors expressed on DC plasma membrane. The final stages of maturation are contributed by T lymphocytes, and are mediated by direct cell-to-cell contact and cytokines secretion, through CD40-dependent and -independent pathways (Bell et al., 1999).

The maturation process is characterized by changes in the efficiency of antigen uptake, intracellular transport and degradation, and in the intracellular trafficking of MHC molecules (Thery et al., 2001). In particular, DCs lose antigen-capturing ability, while peptide loading, as well as the half-life and delivery to the cell surface of MHC molecules, is increased. Surface expression of T cell costimulatory molecules also

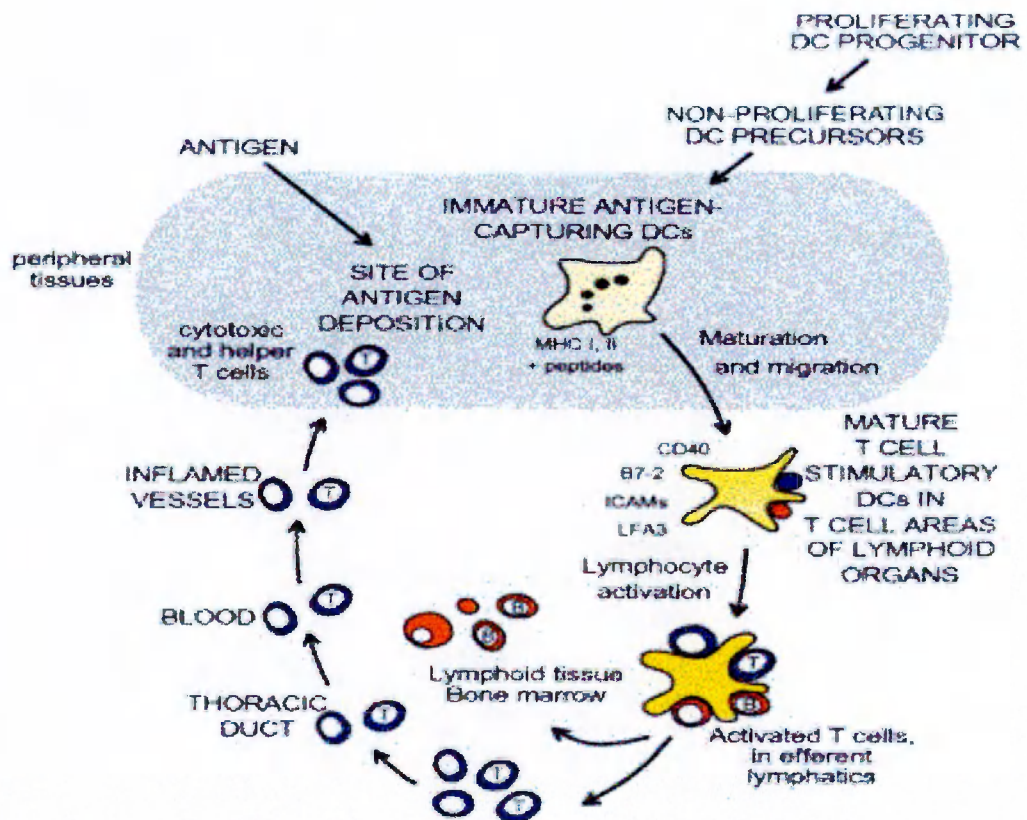
increases. These molecular events explain how DCs become potent APCs, capable of activating naive T lymphocytes and initiating adaptive immune responses.

To interact with T cells, however, DCs also need to migrate out of the tissues to reach secondary lymphoid organs. Thus, concomitant to the modifications of their antigen presentation abilities, maturation also induces massive migration of DCs out of peripheral tissues (Banchereau et al., 2000). The basis for migration resides in the modified expression of chemokine receptors and adhesion molecules together with profound changes of the cytoskeleton organization. The scheme shown as figure 1 highlights the most important features of DCs. By linking antigen uptake, peptide loading, and cell migration to the encounter of a danger signal (Gallucci et al., 2001), these cells restrict presentation to those antigens internalized during maturation, thus favoring stimulation of T cells specific for potentially pathogenic antigens (Guermonprez et al., 2002).

Under different microenvironmental conditions DCs can drive lymphocytes into distinct classes of effectors, critical for resistance to infections and tumors. Conversely, DCs can generate regulatory T cells that suppress activated T cells, a function of importance in autoimmunity and transplant rejection. Moreover, DCs are involved in innate immunity, they can respond to microbial challenge by producing substantial amounts of cytokines, such as IL-12 and interferons, which play a critical role in host defense. Dendritic cells also activate NK cells that rapidly kill select targets and also produce cytokines relevant to innate immune responses. Finally, it is becoming increasingly clear that DCs also capture antigens against which immunity is normally avoided. These include environmental proteins chronically found in the respiratory and digestive tracts, as well as self antigens derived from tissues exhibiting constitutive cell

turnover. The capture of these proteins in the absence of microbial perturbations allows DCs to control tolerance to self and “normal” environmental constituents (Mellman et al., 2001).

Figure 1 The life cycle of DCs



Antigens are captured by DCs in peripheral tissues and processed to form MHC-peptide complexes. As a consequence of antigen deposition and inflammation, DCs begin to mature and migrate to lymphoid organs where, after maturation, they display peptide-MHC complexes, which allow selection of rare circulating antigen-specific lymphocytes. Activated T lymphocytes migrate and can reach the injured tissue. Helper T cells secrete cytokines, which permit activation of macrophages, NK cells and eosinophils. Cytotoxic T cells eventually lyse the infected cells. B cells, after contact with T cells and DCs, mature into plasma cells, which produce antibodies that neutralize the initial pathogen (from Banchereau and Steinman 1998).

3.2 The dendritic cell: development and subsets

The diverse functions of DCs in immune regulation depend on the plasticity of DCs at the immature stage as well on the diversity of DC subsets and lineages. Immature DCs (imDCs) are continuously produced within the bone marrow from hematopoietic stem cells. FLT-3 ligand, and to a lesser extent GM-SCF, represent the key factors for DC growth and differentiation in vivo (Pulendran et al., 2001). In the bone marrow $CD34^+$ hematopoietic stem cells differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) (Fig 2). $CD34^+$ CMPs appear to differentiate into $CD34^+ CLA^+$ and $CD34^+ CLA^-$ populations, which subsequently differentiate into $CD11c^+ CD1a^+$ and $CD11c^+ CD1a^-$ imDCs, respectively. While $CD11c^+ CD1a^+$ imDCs migrate into the skin epidermis and become Langerhans cells, $CD11c^+ CD1a^-$ imDCs migrate into the skin dermis and other tissues, and become interstitial imDCs (Liu 2001).

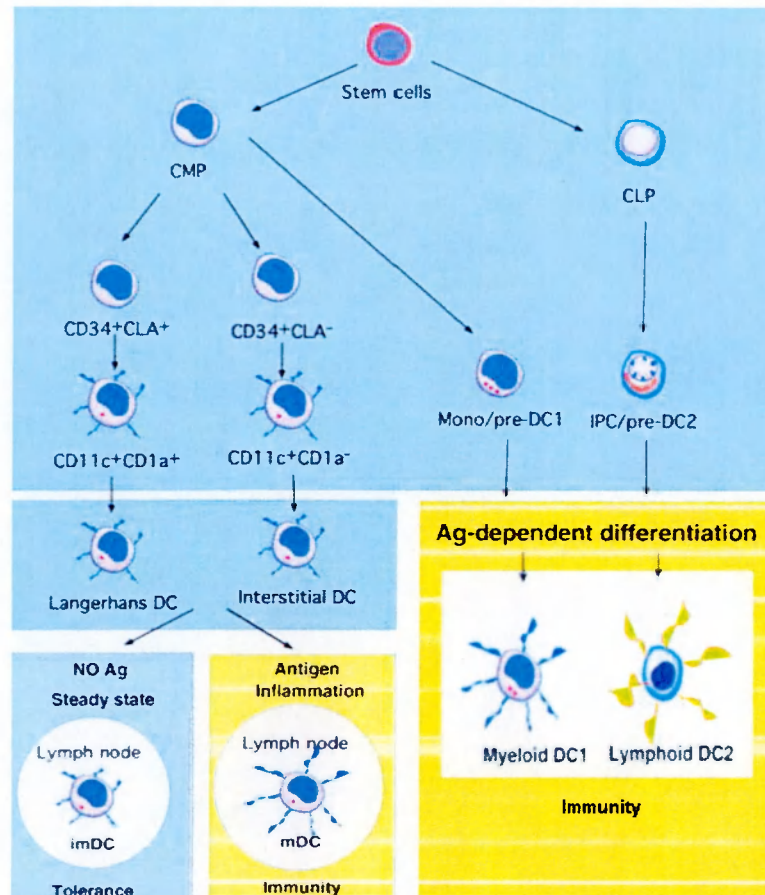
The $CD34^+$ stem cells-derived Langerhans cells and interstitial imDCs display different phenotypes and function (Caux et al., 1997). For example, interstitial imDCs, but not Langerhans cells take up large amounts of antigens by the mannose receptors and to produce IL-10, which may contribute to naïve B cell activation and IgM production in the presence of CD40 ligand and IL-2. Langerhans cells, on the other hand, induce $CD8^+$ T cell-priming higher than interstitial DCs. Since in the human fetus and in newborn rats imDCs are present in lymphoid and nonlymphoid tissues, their production and diversification from $CD34^+$ stem cells in the bone marrow appear to be a part of hematopoiesis, which is pathogen-independent and occurs in a steady-state. Based on the steady-state migration into both nonlymphoid and lymphoid tissues, and

the ability to actively sample self-antigens, it has been proposed that imDCs play critical roles in peripheral tolerance by inducing CD4⁺ and CD8⁺ regulatory T cells (Liu 2001).

In addition to the two subsets of imDCs, stem cells also give rise during hematopoiesis to two types of DCs precursors (pre-DCs): monocytes (pre-DC1) and plasmacytoid cells (pre-DC2). ImDCs, but not pre-DCs, have dendrites or veils, exhibit high mobility in culture, and colonize nonlymphoid tissues in the absence of stimulation. ImDCs express moderate levels of costimulatory molecules and induce moderate T cell activation in vitro. By contrast, isolated pre-DCs express very low levels of costimulatory molecules and do not induce significant naïve T cell activation in culture (Liu 2001). When kept in culture with GM-CSF and IL-4, or after phagocytosis of bacteria pre-DC1s differentiate into immature myeloid DCs (im-DC1s). On the other hand, when in culture with IL-3, or following an innate immune response to viral stimulation pre-DC2s differentiate into im-DC2s.

DCs derived from pre-DC1s and pre-DC2s cultured with GM-CSF plus IL-4, and IL-3, respectively, display different functional properties after CD40 ligand activation. DC1s produce large amount of IL-12 and induce strong T helper type I (Th1) and cytotoxic T lymphocyte (CTL) responses, while DC2s do not produce large amount of IL-12, and induce Th2 responses, or the generation of IL-10-producing CD8⁺ T suppressor cells (Rissoan et al., 1999; Ito et al., 2001). Unlike IL-3- and CD40 ligand-induced DC2s, which promote Th2 responses, viral-induced DC2s promote helper T cells to produce both IFN- γ and IL-10.

Figure 2 DCs development from hematopoietic stem cells



CD34⁺ hematopoietic stem cells differentiate into common myeloid progenitors cells (CMP) and common lymphoid progenitors cells (CLP). The CMPs differentiate into CD34⁺ CLA⁺ and CD34⁺ CLA⁻ late progenitor cells. While CD34⁺ CLA⁺ cells differentiate into CD11c⁺ CD1a⁺ Langerhans cell precursor, CD34⁺ CLA⁻ cells differentiate into CD11c⁺ CD1a⁻ interstitial DC precursors in blood. The blood CD11c⁺ CD1a⁺ Langerhans cell precursor migrate into the skin epidermis and became Langerhans cells and the CD11c⁺ CD1a⁻ cells migrate into the skin dermis and other tissues to become interstitial DCs. The above processes of DCs development and diversification are antigen-independent. Without antigen/pathogen stimulation, both Langerhans cells and interstitial DCs may undergo a steady-state migration into the draining lymph nodes, where they may play a critical role in immuno tolerance. Upon microbial invasion and inflammation, Langerhans cells and interstitial DCs rapidly migrate into the draining lymph nodes. They undergo maturation and initiate primary immune response. CMP and CLP also give rise to myeloid pre-DC1s and lymphoid pre-DC2s in bone marrow. During bacterial and viral infection, pre-DC1s and pre-DC2s differentiate into DCs and initiate respectively antibacterial and antiviral adaptive immune responses (modified from Liu 2001).

3.3 Induction of dendritic cell maturation and signal transduction

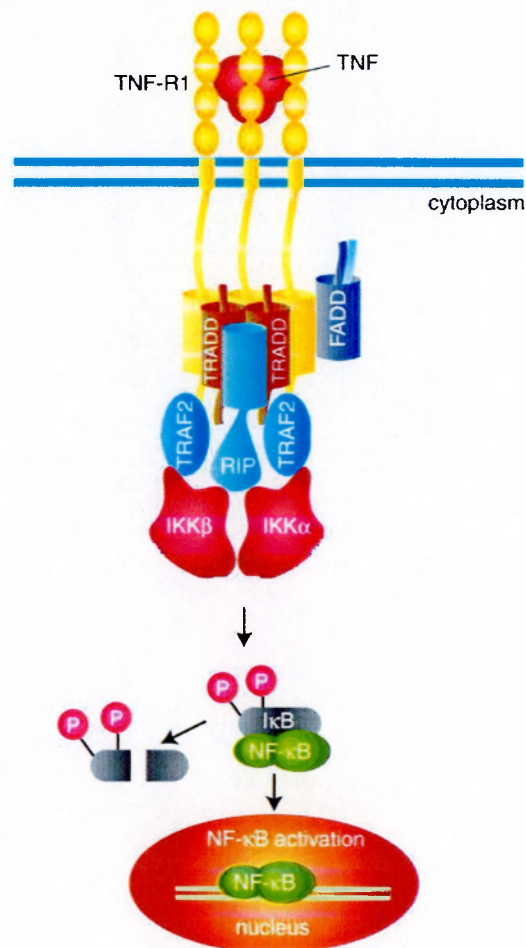
Dendritic cells can be activated to enter the maturation process in the presence of two types of signals: direct recognition of pathogens (through specific pattern-recognition receptors) and indirect sensing of infection (through inflammatory cytokines and internal cellular compounds) (Gallucci et al., 2001). Five types of surface receptors are reported to trigger DCs maturation: (i) cytokine receptors, (ii) Toll-like receptors (TLR), (iii) receptors activated by ligands expressed on T cells, (iv) FcR, and (v) sensors for cell death.

(i) *Maturation by cytokine receptors*: DCs sense danger and infections indirectly, through inflammatory mediators such as TNF- α and IL-1 β , whose secretion is triggered by pathogens (Bell et al., 1999; Kalinski et al., 1999). Most of the receptors which trigger DC maturation are known to be expressed also in other cell types, where their signal transduction cascades have been analyzed in detail. I will describe a few general processes and then concentrate mainly on studies which specifically analyzed DC signal transduction.

TNF- α signaling occurs through two distinct cell surface receptors, TNF-R1 and TNF-R2. In vitro DC differentiation is triggered by activation of p55 TNF-R1 (Sallusto et al., 1995). The initial step involves binding of the TNF- α trimer to the extracellular domain of TNF-R1 with release from the intracellular domain (ICD) of TNF-R1 of the inhibitory protein silencer of death domains (SODD). Aggregated ICD is recognized by the TNF receptor-associated death domain (TRADD), which recruits additional adaptor proteins, i.e. receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2), and the Fas-associated death domain (FADD) (Chen et al., 2002). These latter proteins recruit to TNF-R1 various enzymes that are responsible for initiating signalling events

(Fig 3). In addition, two major transcription factors, NF- κ B and c-Jun, are activated. In the case of NF- κ B this involves the phosphorylation by the I κ B Kinase complex (including IKK α and IKK β), of the inhibitor I κ B, allowed by its ubiquitination and degradation by proteasomes. As a consequence, NF- κ B then translocates to the nucleus and participates in the expression of genes necessary for DC maturation (Banchereau et al., 1998).

Figure 3 TNF- α signal transduction pathway

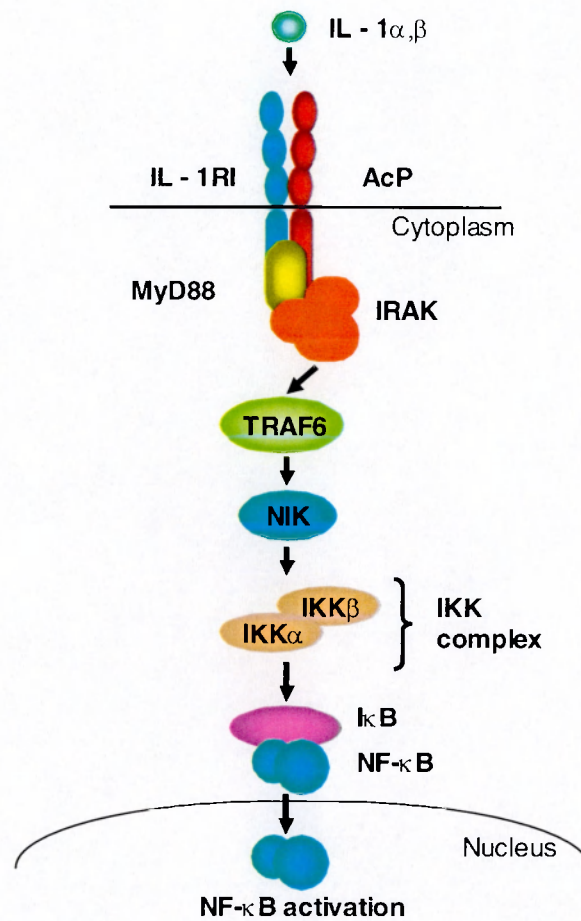


Engagement of TNF- α with its cognate receptor TNF-R1 results in the release of SODD and formation of a receptor-proximal complex containing the important adaptor proteins TRADD, TRAF2, RIP, and FADD. These adaptor proteins in turn recruit additional key pathway-specific enzymes to the TNF-R1 complex, where they become activated and initiate downstream events leading for example to NF- κ B activation (modified from Chen and Goeddel 2002).

The IL-1R system is composed of a ligand-binding subunit, IL-1RI, and a signal transducing subunit, IL1RacP. Upon binding of IL-1, IL-1RI forms a complex with IL1RacP. The adaptor protein, MyD88, is next recruited to this complex, which in turn facilitates its association to the IL-1R-associated kinase (IRAK), via its death domain. After autophosphorylation, IRAK dissociates from the receptor complex and interacts with TRAF6. Association of TRAF6 with NF- κ B-inducing kinase (NIK, a MAP 3 kinase-related protein) leads to the activation of the IKK complex with ensuing direct phosphorylation of I κ B, followed by dissociation and degradation of this inhibitory component from NF- κ B.

Human DCs respond not only to TNF- α , but also to IL-1 β and CD40-L with intracellular ceramide accumulation, as they are induced to differentiate. The ceramide-mediated pathway can shut down antigen capture by the DCs (Sallusto et al., 1996).

Figure 4 IL-1R signalling pathway



Upon binding of IL-1, IL-1RI forms a complex with IL1RacP. This complex recruits IRAK via an adaptor MyD88. IRAK then becomes autophosphorylated, dissociates from the receptor complex, and interacts with TRAF6. TRAF6 associates with NIK. NIK activates the I κ B kinase complex (including IKK α and IKK β) that directly phosphorylates I κ B, which allows dissociation and degradation of this inhibitory component of NF- κ B (modified from Akira 2001).

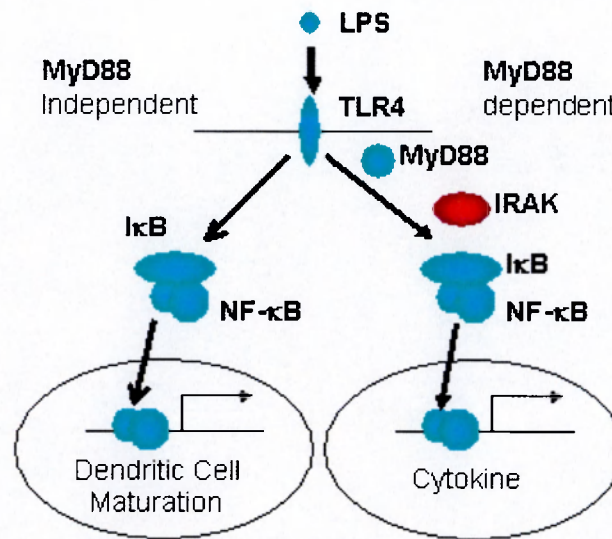
(ii) *Maturation by TLR*: DCs mature in response to various pathogenic compounds, including several bacterial wall components (such as lipopolysaccharide, LPS), unmethylated CpG motifs, and double-stranded RNA. Most of these molecules are recognized by a large family of surface receptors called TLR (Medzhitov et al., 2000). In different cells of the immune system, pathogen-associated pattern molecules are specifically recognized by one or by a combination of TLRs. For example, through binding of LPS TLR4 mediates the responses to GRAM⁻ bacteria; TLR2 is involved in the responses to various GRAM⁺ cell wall components (including bacterial peptidoglycans), and to bacterial lipoproteins; TLR5 recognizes flagellin from both GRAM⁺ and GRAM⁻ bacteria; and TLR9 binds unmethylated CpG motifs (Aderem et al., 2000).

Dendritic cells express a subset of receptors, including TLR2, TLR3, and TLR4 (Visintin et al., 2001; Muzio et al., 2000). The function of TLR2 has been characterized. It triggers DC maturation in response to bacterial peptidoglycans (Michelsen et al., 2001), lipopeptides (Hertz et al., 2001), and mycoplasma lipoproteins (Nishiguchi et al., 2001). TLR3 is expressed exclusively by DCs, and absent from precursors monocytes. Moreover, the expression of TLR3 increases dramatically during monocyte differentiation and decreases significantly after treatments inducing maturation. The TLR family is characterized by a cytoplasmic domain with high homology to the intracellular portion of the IL-1R family. Signalling of human TLRs occurs by the same transduction molecules involved in signalling through IL-1R. For example, TLR4 engagement results in the recruitment of an adaptor molecule, MyD88, followed by the kinase IRAK and the subsequent steps through TRAF6 and NIK, leading to NF- κ B activation (Akira 2001). Rescigno et al. showed that LPS-induced maturation of DCs

involves two signalling pathways: (a) ERK kinase for DC survival, and (b) NF- κ B for processes involved in DC maturation: increased expression of costimulatory and MHC-class II molecules, release of chemokines, and migration (Rescigno et al., 1998). More recently, Ardeshtna et al., confirmed that NF- κ B is required for LPS-induced maturation of human monocyte-derived DCs (Ardeshtna et al., 2000). They also found that survival of these DCs depends on activation of the PI3K. Finally, recent results identify MyD88 as an essential component of TLR signalling in DCs (Kaisho et al., 2001).

Not all TLRs, however, activate a MyD88-dependent signalling pathway. In the absence of MyD88, DC maturation induced by TLR2 and TLR9 is completely abolished, whereas maturation by TLR4 activated by LPS is maintained (Muzio et al., 1998; Kaisho et al., 2001). A MyD88-independent pathway, involving MAPK and NF- κ B, exists therefore downstream of TLR4 (Muzio et al., 1998; Akira 2001).

Figure 5 MyD88-dependent and -independent pathways in DC maturation



At least two signalling pathways originate from the cytoplasmic portion of TLR4. One is the MyD88-dependent pathway, which subsequently activates IRAK, TRAF6, and NF- κ B. This pathway is essential for cytokine production. The other is the MyD88-independent pathway, which cannot activate IRAK or TRAF6. Although the signalling molecules involved are unknown, this pathway can also induce NF- κ B activation. This NF- κ B activation cannot lead to cytokine induction but to induction of costimulatory molecules such as CD40, CD80, and CD86 in DCs (modified from Akira 2001).

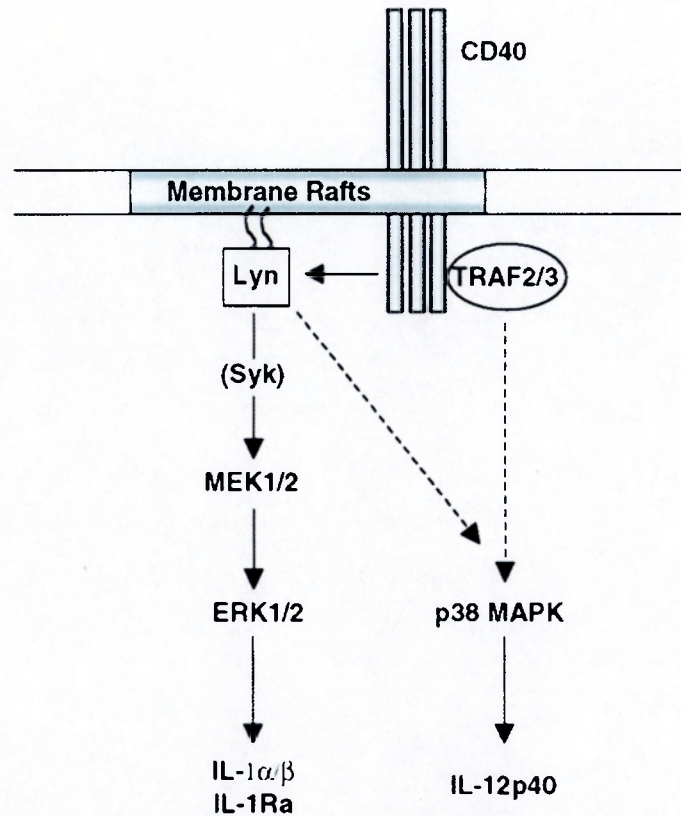
(iii) *Maturation by ligands expressed on T cells.* Triggering of CD40 by CD40L on T cells does induce DC maturation (Schuurhuis et al., 2000; Caux et al., 1994). Most of the studies regarding CD40-mediated signal transduction were carried out by the use of B lymphocytes. Despite the impressive amount of data, however, knowledge of this pathways remains incomplete.

Multimerization of CD40 molecules following engagement with CD40L or antibodies appears as a critical initiating step for CD40-mediated signalling. Although the cytoplasmic domain of CD40 lacks sequences indicative of any catalytic activity, it

can associate with members 2, 3, 5 and 6 of TRAFs. After recruitment to the CD40 cytoplasmic tail following receptor engagement (a step that is enhanced by CD40 oligomerization) TRAFs mediate the activation of NF- κ B and MAPK family. The CD40 cytoplasmic tail also associates constitutively with Jak 3, which becomes activated following CD40 engagement and leads to activation of STATs. In addition, numerous studies support the idea that early events in CD40 signalling involve stimulation of protein tyrosine kinases, including Lyn, Fyn, and Syk (Schonbeck et al., 2001). In DCs CD40 signalling is initiated at plasma membrane cholesterol-rich microdomains (rafts). TRAF2/3 and src-family protein kinases (such as Lyn) are recruited together with CD40 to membrane rafts. TRAF2/3 initiates the activation of p38MAPK, and lyn induces ERK activation (Vidalain et al., 2000). p38 MAPK is required for CD40-induced IL-12 production in DCs because these cells derived from Mkk3 (a p38 activator)-deficient mice fail to produce IL-12 in response to CD40 stimulation (Lu et al., 2001) (Fig 6).

T cells can induce DC maturation through CD40-dependent and -independent mechanisms. Indeed, triggering of Fas and OX40L on DCs by FasL and OX40 on T cells respectively may induce functional maturation (Rescigno et al., 2000; Ohshima et al., 1997).

Figure 6 Model of CD40-mediated signalling in human DCs.



Initiation of signalling events requires a membrane raft reorganization triggered by engagement of CD40, which allows Lyn activation and TRAF2 and 3 recruitment. Lyn activation, and possibly that of other Src family kinases, leads to IL-1 α , IL-1 β and IL-1Ra mRNA expression via a MEK/ERK pathway. p38 MAPK activation, which induces IL-12 mRNA expression, is probably stimulated through a TRAF-initiated pathway, and to some extent in the early phase of CD40 signalling, through a Src family kinase-dependent pathway (from Vidalain et al., 2000).

(iv) *Maturation by FcR*: DCs may also be activated during the endocytic process through the engagement of receptors to the Fc portion of immunoglobulins (FcR). In addition, engagement of most FcR, including Fc γ RI and Fc γ RIII, by immune complexes or specific antibodies, induces DC maturation (Geissmann et al., 2001; Jurgens et al., 1995; Regnault et al., 1999). This process requires the FcR-associated γ -chain that contains an immunoreceptor tyrosine-containing activation motif (ITAM). Tyrosine

phosphorylation of this motif may initiate DC maturation. FcεRI triggering in Langerhans cells induces phosphorylation of the tyrosine kinase syk and Ca⁺⁺ mobilization. It has been recently demonstrated that FcγR engagement in mouse DCs induces syk and ERK phosphorylation, and that syk is necessary to immune complex induced-DC maturation (Guermonprez et al., 2002).

(v) *Maturation by cell death*: cell death can be sensed as a danger signal by DCs. Shi et al. showed that cell injury releases adjuvant compounds that enhance T cell responses (Shi et al., 2000). Two studies showed that necrotic, but not apoptotic, cell death induces mouse and human DC maturation in vitro (Sauter et al., 2000; Gallucci et al., 1999), although another study found that apoptotic bodies may induce DC maturation (Rovere et al., 1998). One should be careful in interpreting these results, however, because LPS, mycoplasma contamination (Salio et al., 2000) or CD40L expression by apoptotic bodies (Propato et al., 2001) could be responsible for DC maturation. The nature of the DC activating compounds is in fact still unclear.

The list of processes proposed to induce DC maturation is still long. It includes nucleotides, such as ATP and UTP, acting through purinergic receptors (Gallucci et al., 2001) and hsps, including gp96, hsp90, and hsc70, released by necrotic cells, whose mechanism is still unclear (Basu et al., 2000; Singh-Jasuja et al., 2000). Although CD91 has recently been identified as a gp96, hsc70, and hsp90 receptor, its role in DC activation has not yet been explored. Hsp60 and hsc70 activate macrophages through CD14 and/or TLR (Vabulas et al., 2001; Ohashi et al., 2000; Asea et al., 2000; Kol et al., 2000), but receptors engaged on DCs have not yet been characterized. Recent data show that release of high mobility group 1 (HMGB1) protein from necrotic, but not apoptotic cells can induce an inflammatory response, acting as a diffusible signal of

unprogrammed death to bystander immune cells and activating DCs (Scaffidi et al., 2002; Rovere, personal communication).

It is unlikely that all these pathways for the induction of DC maturation are redundant. In addition to result in functionally distinct DC populations, different activation signals may either act synergistically or regulate each other. For example, human TGF- β -untreated DCs are activated by LPS or TNF- α ; this does not occurs with TGF- β -treated monocyte-derived DCs, which instead require a CD40-dependent signal to acquire high T cell stimulating activity (Geissmann et al., 1999).

3.4 Antigen uptake in dendritic cells

Dendritic cells were long belived to display low endocytic activities. Therefore, because of their inability to take up antigens, and in spite of their high MHC class II expression, they were not considered as APCs. This idea lasted until regulation of the endocytic ability of DCs was discovered. Bone marrow-derived DCs at an early stage of development were shown to internalize particulate antigens, an activity that in mature DCs became weak or inactive (Steinman et al., 1995). The establishment of an in vitro system that allows human DCs to be mantained in culture without affecting their immature phenotype, i.e., with efficient antigen uptake and processing, has provided an important tool to gain insights into basic mechanisms governing differentiation (Sallusto et al., 1995). Tissue DCs capture pathogens, infected cells, dead cells, or their derived products to use them for antigen presentation. Interestingly, pathogen recognition and uptake are in many cases accompanied by activation/maturation of DCs (Guermonprez et al., 2002).

3.4.1 Receptor-Mediated Endocytosis

During receptor-mediated endocytosis, the uptake of macromolecules occurs through specialized regions of the plasma membrane, termed coated pits. The process is initiated by a signal in the cytoplasmic tail of stimulated receptors, recognised by a family of adaptors responsible for the recruitment of clathrin lattices and for the formation of clathrin-coated endocytic vesicles (Slepnev et al., 2000). A large number of receptors destined to be endocytised by this procedure are expressed by imDCs.

Mouse imDCs express receptors for immunoglobulins: Fc γ RI, Fc γ RII, and Fc γ RIII, which mediate the uptake of immune complexes or opsonized particles (Esposito-Farese et al., 1995; Fanger et al., 1996). Human monocyte-derived DCs express mainly Fc γ RII and Fc α R (Geissmann et al., 2001). The neonatal MHC class I-like FcR for IgG was also found on human monocytes-derived DCs (Zhu et al., 2001). Another receptor of the immunoglobulin superfamily competent for antigen presentation, named immunoglobulin-like transcript (ILT)-3, was found in imDCs. (Cella et al., 1997). Moreover, immature DCs express complement receptor CR3 and CR4, but not CR1 and CR2 (Reis e Sousa et al., 1993).

Heat shock proteins (hsps) derived from tumor cells or infected cells, are known to stimulate antigen-specific T cell responses *in vivo*. Hsp70 and gp96 bind APCs and are internalized through specific membrane receptors (Castellino et al., 2000; Arnold-Schild et al., 1999). Scavenger receptors (SRs) are cell surface glycoproteins defined by their potential to bind chemically modified low-density lipoproteins. SRs play an important role in host defense because they are implicated in internalization of various bacteria. Dendritic cells express at least one type of SR; CD36 (class-B-SR), involved in the uptake of apoptotic bodies (Platt et al., 1998).

C-type lectins share a carbohydrate recognition domain and bind ligands in a Ca^{++} -dependent manner. Dendritic cells express several transmembrane C-type lectins, including macrophage-mannose receptor (MMR) (Sallusto et al., 1995) and DEC205 (Jiang et al., 1995). The MMR binds several monosaccharides as well as a wide variety of pathogen antigens, including yeast antigens (Ezekowitz et al., 1991), lipoarabinomannan (Prigozy et al., 1997), and desialylated immunoglobulins (Dong et al., 1999). The MMR is expressed on macrophages and on monocytes-derived DCs, but not on freshly isolated blood monocytes. In humans, DEC205 was only reported on blood DCs, and its function is not yet documented (Kato et al., 2000).

3.4.2 Phagocytosis and Macropinocytosis

Particulate and soluble antigens are efficiently internalized also by phagocytosis and macropinocytosis, respectively. Both processes are actin-dependent, require membrane ruffling, and result in the formation of large intracellular vacuoles. Phagocytosis is generally receptor-mediated, whereas macropinocytosis is a cytoskeleton-dependent type of fluid-phase endocytosis. The engagement of specific receptors initiating phagocytosis triggers a cascade of signal transduction, which is required for actin polymerization and effective engulfment. In general, the same receptors mediate both phagocytosis and clathrin-dependent endocytosis (Guermonprez et al., 2002). Immature DCs were reported to phagocytose almost any bacteria (Bell et al., 1999), yeast cells (d'Ostiani et al., 2000), parasites such as *Leishmania major* (Gorak et al., 1998), apoptotic and necrotic bodies (Subklewe et al., 2001). In macrophages and epithelial cells, macropinocytosis is transiently induced by growth factors and phorbol esters. In imDCs, in contrast, macropinocytosis is constitutive (Sallusto et al., 1995).

Macropinocytosis represents a critical antigen uptake pathway allowing DCs to sample rapidly and non specifically large amounts of surrounding fluid.

3.4.3 Regulation of endocytosis in dendritic cells

Efficient antigen internalization is a specific property of imDCs. During maturation, DCs downregulate their endocytic capacity, thus limiting the range of antigens they are able to present after residing within peripheral tissues. Loss of internalization processes occurs through two independent mechanisms: downregulation in cell surface expression of most antigen receptors (MMR/FcR) and decrease in both macropinocytosis and phagocytosis (Sallusto et al., 1995; Sallusto et al., 1994). Two independent groups have analyzed the molecular mechanism responsible for regulation of endocytosis. They both found that inactivation of the small GTPases cdc42 and rac1 blocks both macropinocytosis and phagocytosis in imDCs (West et al., 2000; Garrett et al., 2000). In contrast to the findings of Garrett et al., West et al. could neither detect any changes in the active forms of cdc42 during maturation nor restore macropinocytosis in mature DCs by microinjecting constitutively active cdc42. These results suggest that DCs express various mechanisms for downmodulation of internalization.

3.5 Chemokines and the migration of dendritic cells

Since this topic is not a subject of my thesis work, it will be described only briefly. The function of DCs is intimately connected to their capacity to migrate. Immature DCs are recruited to the sites of infection where they are exposed to the stimuli that induce migration via afferent lymph to the T cell areas of secondary

lymphoid organs. These changes in migratory behavior have been related to changes in the expression of adhesion molecules and chemokine receptors that allow the cells to sense chemotactic gradients. For instance, when epidermal Langerhans cells are activated and migrate from the skin, E-cadherin is down-regulated and laminin receptors up-regulated (Sallusto et al., 1998).

Chemokines can be broadly divided into inflammatory and constitutive. The first are induced, or strongly upregulated in peripheral tissues by inflammation. The latter have housekeeping functions and may be involved in constitutive leukocyte traffic. The capacity to respond to chemokines depends on the set of chemokines receptors expressed by leukocytes. In DCs rapidly changing their migratory behaviour these receptors are tightly regulated. Immature cells express chemokine receptors, such as CCR1, CCR2, CCR5 and CCR6, which guide them to the site of inflammation. Maturing DCs downregulate expression of inflammatory chemokine receptors and upregulate expression of CCR7 (Sallusto et al., 1998). Up-regulation of CCR7 is relevant for homing of mature DCs, since the known ligands for this receptor, secondary-lymphoid-tissue chemokine (SLC) and the EBL1 ligand chemokine (ELC) are produced in secondary lymphoid organs (Sallusto et al., 1998; Gunn et al., 1998). Recently, MDR-1-type p-glycoprotein (MDR-1) has been identified as a potential mediator of DC migration from the skin to the lymphatic vessels. The mechanism by which MDR-1 acts is still unclear. Recent data suggest in fact that MDR-1 is not a mediator of adhesion. Its activation might therefore be indirect. Based on its well-known role as a membrane transporter, MDR-1 could induce translocation of a soluble substrate, as yet unidentified, that regulates migration (Randolph et al., 1998).

3.6 T cell stimulation by dendritic cells

Maturation is associated with a rapid relocation of antigen-bearing DCs to the T cell zone of secondary lymphoid organs. Maturation also enhances surface levels of costimulatory and adhesion molecules among which important ones are B7.1 and B7.2, which bind both CD28 and CTLA-4 on T cells. These modifications increase the T cell priming ability of DCs. Two signals are necessary in order to achieve full T cell activation: recognition of MHC-peptide complexes on DCs by Ag-specific TCRs, which constitutes "signal one" and interaction between costimulatory molecules expressed by DCs and their ligands expressed by T cells, the so-called "signal two". CD86 on DCs is so far the most critical molecule for amplification of T cell responses. On the other hand, CD28 is constitutively expressed on T cell and facilitates the initial phases of T cell priming, while CTLA-4 is inducible and is involved in the downregulation of T cell responses (Banchereau et al., 2000).

T cells can activate DCs via CD40 ligand (CD40-L)-CD40 signalling, leading to increased expression of CD80/CD86 and cytokine release (IL1, TNF, chemokines, and IL-12) (Sallusto et al., 1994; Ridge et al., 1998; Schoenberger et al., 1998; Caux et al., 1994). Triggering of CD40 on DCs results in upregulation of OX40 ligand (Stuber et al., 1995). Ligation of OX40 at the time of priming induces T cells to migrate to B cell areas (Brocker et al., 1999). At least in vitro, ligation of OX40 to CD4⁺ T cells induces upregulation of the chemokine receptor CXCR-5 on naïve CD4⁺ T cells (Flynn et al., 1998). The ligand for this chemokine receptor is expressed in B cell follicles, which explains why OX40-activated T cells accumulate at this site (Gunn et al., 1998).

Mature DCs also express 4-1BB ligand (DeBenedette et al., 1997), which complements the function of OX40-L. 4-1BB is a costimulator expressed primarily on

activated CD4⁺ and CD8⁺ T cells (Saoulli et al., 1998). 4-1BB costimulatory signals preferentially induce CD8⁺ T cell proliferation and production of IFN (but not of IL-4) (Kim et al., 1998), leading to the amplification of in vivo cytotoxic T cell responses in graft-vs-host disease as well as in allograft rejection (Shuford et al., 1997). Whether OX40-L and 4-1BB are expressed simultaneously by the same DC or separately by distinct DCs remains to be established.

Engagement of RANK, a member of the TNFR family, by its ligand (RANKL/TRANCE) expressed on activated T cells, stimulates secretion of cytokines like IL-1, IL6, and IL-12 by DCs. As a whole, this process results in increased DC survival by inhibition of apoptosis and, in turn, in enhanced proliferative T cell responses in mixed lymphocyte reactions. The demonstration that TRANCE is responsible for the CD40L-independent T-helper cell activation during viral infection suggests an important and specific role for this molecule during infection (Anderson et al., 1997; Wong et al., 1997; Josien et al., 1999).

Dendritic cell-T cell clustering is mediated by several adhesion molecules, like integrins $\beta 1$ and $\beta 2$ and members of the immunoglobulin superfamily (CD2, CD50, CD54, and CD58) (Hart 1997; Bell et al., 1999). Another important accessory molecule is DC-SIGN, i.e. the first recognized DC-restricted product that helps stimulate resting T cells. It has been proposed that the interaction of DC-SIGN with ICAM-3 on T cell can mediate the loose adhesion that takes place between DCs and T cells in the apparent absence of foreign antigen. Such adhesion provides the TCR an opportunity to scan the DC surface, searching for the small amounts of MHC-peptide ligands, which then activate resting T cells. DC-SIGN allows the DCs to interact temporarily with naïve T cells.

Whichever the signalling molecules involved, antigen recognition leads to the formation of a contact zone termed the immunological synapse, rich in interacting adhesion and signalling molecules (Steinman 2000).

3.7 Nitric Oxide and its synthesizing enzymes

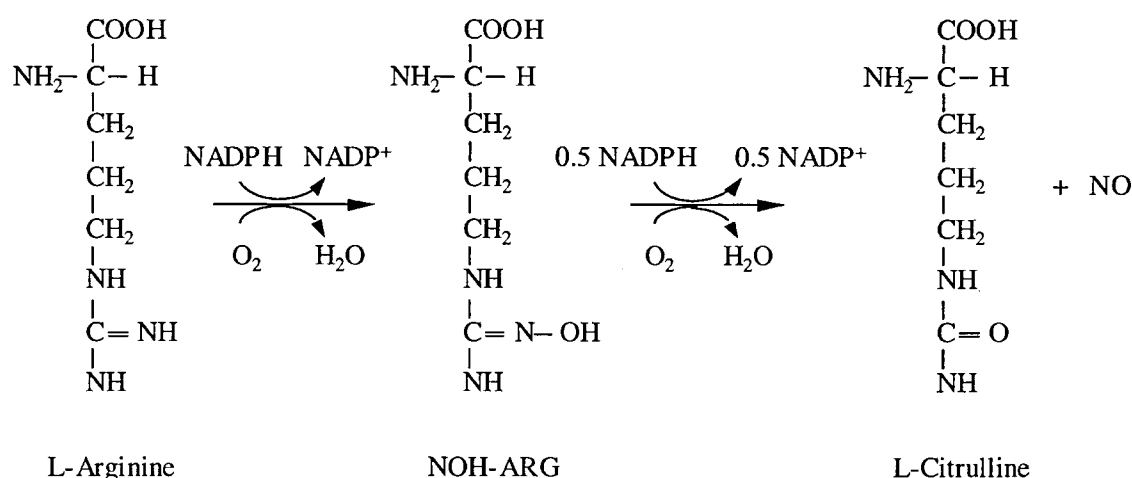
After providing a general picture of the DCs role in immunological responses and of their ligand-receptor regulation time has come to introduce a new participant of the game, nitric oxide (NO) which is the major actor of my thesis.

Nitric oxide is a free-radical gas that readily diffuses through cells and cell membranes where it reacts with molecular targets. Contrary to conventional biosignalling molecules that act only by binding to specific receptor molecules, NO induces its biological actions also via a wide range of chemical reactions. With its molecular weight of 30, NO is a small molecular mediator (Fang 1997), it has a short half life, between 3 and 20 seconds in aqueous and oxygen-containing solutions (Vladutiu 1995). Nitric oxide is a double-edge sword. Produced by mammalian cells in an appropriate quantities and time, it is a key signalling molecule in physiological process as diverse as host-defense, neuronal transmission, and vascular regulation (Nathan 1992; Nathan et al., 1994; Stuehr et al., 1992). On the other hand, excessive NO synthesis has been implicated as a causal or contributing factor to pathophysiological conditions including many human diseases: vascular shock, stroke, diabetes, neurodegeneration and chronic inflammation (MacMicking et al., 1997).

Responsible for the synthesis of NO is a family of enzymes: the nitric oxide synthases (NOSs), which catalyze the conversion of L-arginine to NO and L-citrulline. Nitric oxide synthase produces NO by oxidizing guanidino nitrogen of L-arginine,

utilizing molecular oxygen and NADPH as cosubstrates. Two molecules of water are coproducts. This process involves two successive monooxygenation reactions, with the initial reaction yielding N ω -hydroxy-L-arginine as an isolatable intermediate (Stuehr et al., 1991) and the final reaction processing the hydroxylated nitrogen of NOH-ARG to NO (Stuehr et al., 1992). All NOS isoforms contain four prosthetic groups: flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), iron protoporphyrin IX (heme), and tetrahydrobiopterin (THB). The NOS reaction is summarized in Fig. 7.

Figure 7 Biochemical pathway of NO production in mammalian cells. Two sequential reactions are catalyzed by NO synthase.



NOS catalyze the NADPH- and O₂-dependent five electron oxidation of L-arginine to NO and citrulline. When fully active the enzyme contains one equivalent each of FAD, FMN, heme, THB per monomer. The reaction sequence shown in this equation represents two successive mono-oxygenase reaction: NOH-ARG is a tightly bound intermediate and its formation from L-arginine requires 1.5 equivalents of NADPH, the successive monooxygenase reaction from NOH-ARG to citrulline and NO requires 0.5 equivalents of NADPH. In both steps the four electrons necessary to reduce each O₂ are derived from both NADPH and the aminoacid substrate. In the NOS reaction two molecules of water are coproducts (from Griffith and Stuehr 1995).

Mammalian cells are endowed with three genes encoding distinct isoforms of NOS. Two NOS are constitutively expressed: endothelial NOS (eNOS) in endothelial cells, and neuronal NOS (nNOS) in neurons and various other cell types including kidney macula densa cells, B-pancreatic cells, skeletal muscle, and epithelial cells of the lung, stomach, and uterus. Synthesis of NO by macrophages is mediated by the third species of this enzyme, the inducible NOS (iNOS), not expressed by resting cells and induced by a variety of immunological stimuli, as I will extensively describe in a following chapter (see chapter 3.9) (Gross et al., 1995). Although all NOS isoforms require bound calmodulin for activity, only iNOS has high affinity for calmodulin which remains stably bound, thereby conferring iNOS its full catalytic activity even at the low basal calcium concentration of resting cells (Cho et al., 1992). It is thought that eNOS and nNOS produce small, physiological concentration of NO in response to transient elevation in intracellular calcium, whereas iNOS, when induced, produces large and continuous fluxes of NO until substrates become limiting.

In 1991, the cDNA sequence coding for rat cerebellar nNOS was first identified (Bredt et al., 1991). The C-terminal portion of nNOS and all subsequently studied NOS isoforms show partial homology to NADPH cytochrome P450 reductase, the only other mammalian enzyme known to bind both FAD and FMN. Consensus sequences for NADPH, FAD, and FMN binding sites were identified within the C-terminal region NOS. cDNA and predicted amino acid sequences for eNOS and iNOS have also been reported (Fig 8). Regions of high homology are associated with cofactor binding sites. In the N-terminal part of the protein lies a region where the three types of NOS share 65-71% sequence identity. This region contains the putative L-arginine-binding region and probably also the binding sites for tetrahydrobiopterin and heme. The binding site

and the reductase (C-terminal) domain, with sequence homology to cytochrome P450 reductase (Sheta et al., 1994; Griffith et al., 1995). Calmodulin-binding region acts as a hinge between the C-terminal reductase and the N-terminal oxygenase domains. When Ca^{2+} /calmodulin is not bound, the domains are not aligned, and the reductase domain can no longer supply electrons to heme. When Ca^{2+} /calmodulin is bound, the domains align and the enzyme is active (Abu-Soud et al., 1993). This model represents a mechanism for nNOS and eNOS activation by increased intracellular $[\text{Ca}^{2+}]$ and also accounts for the insensitivity of iNOS to changes in $[\text{Ca}^{2+}]$ because calmodulin is tightly bound to that isoform and does not dissociate. In iNOS, the domains are always aligned, and the enzyme is permanently active.

3.8 Evidence for involvement of nitric oxide synthase in immune responses

The antimicrobial activity of NO was demonstrated by a variety of approaches. Here I will describe the most relevant ones.

3.8.1 Correlation of iNOS expression and nitric oxide production with host response to infection

Inducible NOS expression is stimulated by microbial products, as well as by proinflammatory cytokines produced during immune responses. Infections in humans and experimental animals are often associated with significant increases in systemic NO production, revealed by measurement of nitrite and nitrate (NO end-products) in plasma and urine (Anstey et al., 1996; Evans et al., 1993; Ochoa et al., 1991). Increased iNOS expression and NO production can be demonstrated at sites of infection in animal

models such as toxoplasmosis and leishmaniasis (Stenger et al., 1996), or in human infections such as tuberculosis (Nicholson et al., 1996). In cell lines and mice, resistance to microbial growth is often associated with expression of iNOS. Replication of *ectromelia virus*, for example, was restricted in IFN γ treated NO-producing mouse macrophages, yet it was unhindered in fibroblasts or epithelial cells in which IFN γ does not induce iNOS (Karupiah et al., 1993). *Leishmania major* was killed more efficiently by cytokine-stimulated macrophages from mouse strains resistant rather than susceptible to cutaneous leishmaniasis, a distinction reflected in their respective expression of iNOS (Liew et al., 1991). Immunohistochemical appearance of iNOS in macrophages of resistant C57BL/6 mice was associated with the disappearance of *Leishmania major* from cutaneous lesions and draining lymphonodes (Stenger et al., 1994).

3.8.2 Decreased host resistance upon treatment with inhibitors of NOS and disruption of iNOS alleles

More direct evidence for the role of NOSs was obtained after the identification of various compounds that inhibit this class of enzymes. These inhibitors were shown to worsen the course of diseases caused by a wide array of pathogen: viruses, bacteria, fungi, protozoa and helminths (MacMicking et al., 1997).

The pharmacological inhibitors used to block iNOS may also be active to some extents on the other NOSs. Moreover, they may have effects unrelated to the inhibition of NOS. Therefore, mice deficient in iNOS (iNOS^{-/-}) were developed to better clarify the function of this enzyme with respect to that of the other gene family members (MacMicking et al., 1995; Wei et al., 1995), since such mice completely lack expression

of iNOS, but not of eNOS and nNOS. Inducible NOS mutant mice are viable, fertile and without evident histopathological abnormalities; activation and the respiratory burst of macrophages, lymphocytes development, and leucocytes migration are however unimpaired. Compared to wild-type and heterozygous mice, iNOS^{-/-} mice show higher susceptibility to *Leishmania major* infection (Wei et al., 1995) and their survival is reduced upon infection with *Listeria monocytogenes* (MacMicking et al., 1995). The same effect has been observed with various other pathogens. When examined, decreased survival correlated with major increases in microbial burden in the affected organs (MacMicking et al., 1997). An important issue addressed through the use of iNOS^{-/-} mice is the pathogenesis of septic shock. Excessive NO synthesis within blood vessel wall has been implicated as the basis for septic- and cytokine-induced circulatory shock, characterized by central blood pressure falls, blood flow maldistribution and myocardium depression. Compared to wild-type, inducible NOS mutant mice were more resistant to vascular collapse and death induced by LPS (Wei et al., 1995; MacMicking et al., 1995).

Immune responses have been investigated also in mice knockout for the other NOS isoforms. An involvement of eNOS and nNOS in immune responses has been shown in studies with nNOS^{-/-} and eNOS^{-/-} mice in two models of colitis, namely dextran sodium sulfate- and trinitrobenzene-induced colities (Mashimo et al., 1999). The role of NO, or better, the lack of its generation by each constitutive isoform, however, is not well defined in these two models. Taken together, these observations indicate a major role for NO generated by iNOS in regulating immune responses, although also NO generated by constitutive isoforms may play a role in specific conditions.

I will therefore concentrate my analysis on specific aspects of regulation of the iNOS enzyme.

3.9 Regulation of inducible nitric oxide synthase

Macrophage iNOS has been isolated as a homodimeric enzyme composed by two identical subunits of 130-kDa (Hevel et al., 1991; Stuehr et al., 1991). Subunit dimerization is required for activities (Baek et al., 1993). Thus macrophage iNOS subunits isolated by gel filtration bind FAD, FMN, and calmodulin but not heme or THB and do not generate NO. Subunit dimerization does not occur spontaneously, but requires the coincident presence of THB, L-arginine and stoichiometric amounts of heme (Baek et al., 1993). Following dimerization, THB and heme, but not L-arginine, remain bound. Thus subunit assembly and cofactor binding appear to be a significant posttranslational modification leading to active iNOS.

Expression of iNOS in macrophages is induced by cytokines and microbial products, primarily at the transcriptional level. Activating cytokines include interferon- γ (IFN γ), interleukin-1 (IL-1) and tumor necrosis factors. Synergism in induction of iNOS activity has been observed with the combination of IFN γ and any of these agents. Cloning and sequencing iNOS gene of the mouse (Lowenstein et al., 1993, Xie et al., 1993) and human (Chartrain et al., 1994) in the 5'-region upstream of exon 1 has revealed the presence of numerous prototypical consensus sequences for genes that are transcriptionally activated by cytokines. The 1749 basepair (bp) upstream flanking region of murine iNOS contains more than 20 identifiable putative sequences for distinct transcriptional activating factors, including two sites for NF-kB that mediate the induction of numerous genes by LPS (Schreck et al., 1992), 10 sites for IFN- γ response

element, 2 sites for IFN- α -stimulated response element, 2 sites for TNF response element, and a binding sites for nuclear factor IL-6, and activating protein-1 (Lowenstein et al., 1993; Xie et al., 1993). Although in iNOS promoter constructs when transfected into macrophages in vitro, only a single NF- κ B site appears to be necessary for activation by LPS (Xie et al., 1993; Xie et al., 1994), induction by LPS was lost or diminished in mice knockouts for genes encoding interferon- γ (Dalton et al., 1993), a subunit of the interferon- γ receptor (Huang et al., 1993), or interferon regulatory factor-1 (IRF-1) (Kamijo et al., 1994). Thus the IFN- γ signalling pathway appears to be crucial for induction of iNOS activity in vivo. In addition to enhancing transcription of the iNOS promoter, IFN- γ may elevate levels of LPS-induced iNOS mRNA via mRNA stabilization (Vodovotz et al., 1993). Other stimuli that induce iNOS activity include cAMP-elevating agents (Koide et al., 1993) UV-B irradiation (Warren 1994) and inhaled ozone (Pendino et al., 1993). Based on the observation that these agents share with LPS the capacity to elicit nuclear translocation of NF- κ B, the latter factor has been proposed as their common mediator (Gross et al., 1995). It is also of interest to note that NO itself exerts a biphasic effect on iNOS transcription. Low concentrations, such those occurring at the onset of macrophage stimulation by cytokines, activate NF- κ B and upregulate iNOS (positive feedback) while high concentrations have the opposite effect. The latter effect may help preventing NO overproduction (Connelly et al., 2001). Moreover, in mesangial cells NO donors can exert stimulatory or inhibitory effects on iNOS expression in a time-dependent fashion (Perez-Sala et al., 2001).

Pharmacological levels of glucocorticoids suppress induction of iNOS. Multiple mechanisms have been proposed, involving a combination of both transcriptional and posttranscriptional effects (Kunz et al., 1996). Interleukin-4 exerted a delayed

suppressive effect on transcription of iNOS (Bogdan et al., 1994). The most potent suppressors known so far in mouse macrophages are however TGF β 1 to 3 (Vodovotz et al., 1994). The physiological importance of this action is shown by the spontaneous expression of iNOS in TGF β 1-deficient mice (Vodovotz et al., 1996). In vitro studies with primary mouse peritoneal macrophages demonstrated that TGF β 1 destabilizes iNOS mRNA, retards the synthesis of iNOS protein, and accelerates its degradation (Vodovotz et al., 1994).

Substrate and cofactor availability are additional important sites for regulation of iNOS activity. In most cell types, uptake of L-arginine is mediated by a family of cationic amino acid transporter proteins (CATs). In macrophages, CAT1 and CAT2 are upregulated by LPS. After stimulation with IFN- γ plus LPS macrophages from CAT2^{-/-} mice showed strong suppression of arginine uptake and NO production. This indicates that arginine transporters and iNOS form a functional unit (Closs et al., 2000; Nicholson et al., 2001). Extracellular arginine concentration is modulated by arginase, an enzyme which degrades arginine to urea and ornithine (Wu et al., 1998). Arginase expression appears to be increased in macrophages by various stimuli, among which are TGF- β s and LPS (Gotoh et al., 1999; Munder et al., 1999). Upregulation of arginase prior to the induction of iNOS prevents NO production by substrate depletion (Munder et al., 1999). When both enzymes are coinduced NO production is not impaired (Fligger et al., 1999), because the K_m value of arginase for arginine is higher than that of iNOS (Wu et al., 1998; Stuehr 1999). Another level of control of NO generation resides in the ability of macrophages to regenerate arginine from citrulline, and thereby to utilize citrulline for the production of NO. Argininosuccinate synthetase, the rate limiting enzyme of the citrulline-NO cycle, is inducible by LPS in the same cells as iNOS (Nussler et al.,

1994). Heme depletion may account for blockade in macrophages that contain normal amounts of iNOS protein (Vodovotz et al., 1994). Another level of post-translational iNOS regulation is represented by guanosine triphosphate cyclohydrolase I, the key enzyme of BH₄ synthesis. In fact, BH₄ can be rate-limiting to NO synthesis (Werner-Felmayer et al., 2002). Expression of this enzyme can be induced or suppressed by cytokines such as IFN- γ , TNF, IL-1 and TGF- β .

A final step regulatory mechanism might be phosphorylation since all NOS isoforms are phosphorylated within cells (Michel et al., 1997; Stuehr 1999). Although serine phosphorylation of eNOS by the Akt kinase is relevant to its activity (Morales-Ruiz et al., 2001), the role of phosphorylation of iNOS under physiological condition remains still little investigated.

3.10 Mechanisms of action of nitric oxide in the immune responses

3.10.1 Direct antimicrobial action of nitric oxide

Over 50 years ago, nitrites were shown to limit bacterial decomposition of meats. The bacteriostatic properties of nitrites were acid-dependent, reflecting the formation of nitrous acid (HNO₂), which dismutates to NO (MacMicking et al., 1997). Numerous bacteria are sensitive to acidified NaNO₂. Such findings may have relevance to the milieu within phagolysosomes of activated macrophages, where low pH could help to catalyze the recovery of NO from nitrite, a waste product of NO oxidation, and where low pH per se may help restrict microbial growth in conjunction with NO or its derivatives (Nathan 1995). A direct action of NO on pathogens has thereafter been demonstrated also by experiments with *L. major* and *L. monocytogenes* (Liew et al.,

1990; Vincendeau et al., 1991). In these studies treatment with NO-generating compounds in the absence of the host cells resulted in inhibition of parasite growth.

Nitric oxide donors can also be toxic to viruses when infecting macrophages. In particular, NO donors have rendered host cells virustatic towards diverse taxons, such as poxviridae (*vaccinia*), herpesviridae (*HSV-1 and EBV-1*), rhabdoviridae (*VSV*), and retro-viridae (*Friend leukemia virus*) (MacMicking et al., 1997).

Although significant progress has been made in identifying interactions between specific cellular constituents and NO-related species, the critical targets responsible for microbial stasis or death by NO are not yet completely clarified. Moreover, significant mechanistic differences appear to exist for the various microbial pathogens. For example, NO[•] itself does not possess antimicrobial activity for *S. typhimurium* or *Escherichia coli* (Pacelli et al., 1995; De Groote et al., 1995), but S-nitrosothiols are bacteriostatic and peroxynitrite is bactericidal for these organisms. In contrast, S-nitrosothiols and NO[•] are microbicidal for *Staphylococcus aureus* (Kaplan et al., 1996) and *L. major* (Assreuy et al., 1994), under conditions in which peroxynitrite does not exert any apparent antimicrobial effect.

Reactive nitrogen intermediates have been shown to modify proteins, DNA, and also lipids. Nitric oxide interactions with proteins can involve reactive thiols, heme groups, iron-sulfur clusters, phenolic or aromatic aminoacid residues, tyrosyl radicals, or amines (Fang 1997). Nitrosylation of cysteine residues is one mechanism by which NO can directly modify a polypeptide, and NO in theory can reduce the activity of an enzyme in case the catalytic site of the latter includes a cysteine residue. Cysteine proteases are critical for virulence or replication of many viruses, bacteria, and parasites. From this point of view the most sensitive condition is that of many viruses

whose genome is translated into a large polyprotein. A viral protease, after excising itself from the polyprotein, cleaves the polyprotein into various components, including RNA polymerase which then can replicate the viral genome. Viral proteases fall into several categories based on their active site residues, including cysteine proteases, serine proteases, and aspartic proteases (Babe et al., 1997). While NO does not inhibit the replication of some viruses that encode serine proteases, such as *alphaviruses*, it can inhibit the replication of many viruses that encode cysteine proteases, like *Coxsackievirus* (Saura et al., 1999) and members of the *Picornavirus* family and the *Coronavirus* family (Mannick et al., 1994; Mannick 1995; Reiss et al., 1998). Inhibition of *Coxsackievirus* replication by NO is due, at least in part, to NO nitrosylation of cysteine residue in the active site of viral protease (Saura et al., 1999). Nitric oxide inactivation of cysteine proteinase could explain the reduction in both viral RNA and viral protein levels, given the characteristics of the *Coxsackievirus* life cycle.

Cysteine proteases are also critical to the replication and virulence of a variety of other micro-organisms, such as *Plasmodium falciparum*, *Plasmodium berghei*, *Leishmania major*, *Schistosoma mansoni*, *Trypanosoma cruzi*, *Streptococcus pyogenes* (Bailly et al., 1992; Eakin et al., 1992; Kumar et al., 1994; Selzer et al., 1997; Wasilewski et al., 1996; Kapur et al., 1993). As an example, malaria parasite uses its cysteine protease falcipain to degrade hemoglobin, to be use as a source of amino acids, and cysteine protease inhibitors block the ability of the parasite to infect erythrocytes and to replicate (Francis et al., 1994; Dominguez et al., 1997). NO inhibits replicaton not only of *Plasmodium*, but of all the above parasites (MacMicking et al., 1997; Reiss et al., 1998). Nitrosylation of cysteine proteases may thus be a general mechanism of

defence against infections by pathogens whose life cycle depends upon cysteine protease activity.

Nitric oxide inhibits the replication also of some viruses that do not encode cysteine proteases, implying the existence of other viral targets of NO. For example, in the nucleus, NO may regulate EBV reactivation through down-regulation of the expression of Zta, the EBV transcription factor that mediates the switch from latent to lytic infections (Rooney et al., 1988). Since Zta induces its own expression (Flemington et al., 1990), NO may down-regulate Zta expression by inhibiting Zta function. Zta is a member of a family that has a cysteine residue within his DNA-binding structure. Under oxidizing conditions, the residue forms an intermolecular disulfide bridge between dimers which inhibits the ability of these proteins to bind DNA (Bannister et al., 1991). Nitric oxide could inhibit Zta function either directly, through S-nitrosylation, or by accelerating the formation of a disulfide bond (Stamler 1994; Lipton et al., 1993). Zta function can also be inhibited indirectly by NO, since NO activates NF- κ B (Lander et al., 1993) and NF- κ B inhibits Zta induced transcription (Gutsch et al., 1994).

Nitric oxide can interact with iron contained in heme proteins, such as guanylate cyclase, which accounts for many of its roles in physiological signal transduction (Murad 1994). In contrast to guanylate cyclase which is activated by NO, other heme proteins, such as catalase and cytochrome oxidase are inhibited by the gas (Kim et al., 1995). Several of these enzymes are located within mitochondria and are involved in the electron transport chain. Therefore, inhibition of respiration may represent, if prolonged, an important cytotoxic action of NO. In aconitase and complexes I, II, as well as cytochrome c oxidase of the respiratory chain NO has been shown to react with the iron-sulphur clusters. The end result of such actions is ATP depletion of target cells and

hence cytotoxicity. Nitric oxide can also inactivate phosphoenolpyruvate carboxykinase and glyceraldehyde-3-phosphate dehydrogenase, both of which are important in glucose metabolism, and thereby reduce the energy production of target cells (Ou et al., 1996).

Several lines of evidence indicate that DNA is an important target of reactive nitrogen intermediates. Some of the effects of NO on DNA may involve impairment of DNA synthesis as well as direct modifications of deoxyribonucleotides. Nitric oxide has an inhibitory effect on vaccinia virus growth through a specific inhibition of the activity of ribonucleotide reductase, which is DNA-synthesizing enzymes (Melkova et al., 1995). Both in vitro and in intact bacteria NO can deaminate DNA, acting via an N-nitrosating intermediate (Routledge et al., 1993; Maragos et al., 1993). NO_2^- and peroxynitrite can also oxidatively damage DNA, resulting in abasic sites, strand breaks, and a variety of other DNA alterations (Maragos et al., 1993).

Nitric oxide has been shown to induce membrane damage, an action mainly sustained by formation of peroxynitrite. Peroxynitrite has been found to mediate lipid peroxidation via a mechanism which does not require iron (Rubbo et al., 1994). Also NO_2^- can induce lipid peroxidation (Halliwell et al., 1992). The relationship between these actions and NO-related antimicrobial activity is unknown.

3.10.2 Indirect antimicrobial action of nitric oxide through modulation of immune cells function

3.10.2.1 *Nitric oxide modulates mast cell functions*

Nitric oxide is not only a direct toxic agent towards infectious organisms, but exerts its function through regulatory effects on many cell types involved in immune responses. Mast cells are highly specialized secretory cells that play important roles in

IgE-mediated allergy and inflammation. After exposure to allergenes mast cells release by exocytosis histamine, heparin and proteases, stored in their dense granules. Inhibition of NO on anti-IgE- and Ca^{2+} ionophore-induced histamine release has been demonstrated in rat and human mast cells (Jorens et al., 1993; Iikura et al., 1998). IFN- γ show similar effects, in fact it inhibits IgE/antigen-induced degranulation and mediator release from mouse and rat mast cells. This action of the cytokine is however indirect since it is far stronger in mixed peritoneal cells compared to purified mast cells preparations. The active intermediate has been shown to be NO (Eastmond et al., 1997). Additionally, mast cells are a rich source of diverse cytokines that are synthesized *de novo* upon cell activation or, as in the case of TNF- α , are stored 'ready-to-go' in the secretory granules. In a rat cell line model a panel of NO donors induced a time-dependent, and in some case reversible, inhibition of IgE/antigen-induced expression of mRNA for TNF- α , IL-4 and IL-6 (Coleman 2002). Nitric oxide inhibits mast cells degranulation only after prolonged incubation times (up to 24 h) before antigen challenge. This 'slow' responsiveness of cultured as well as of primary mast cells is consistent with sustained NO generation by iNOS rather than with transient NO generation by constitutive NOS (Eastmond et al., 1997; Deschoolmeester et al., 1999). Several *in vivo* models demonstrate that NO inhibits various mast cell-dependent processes including histamine-mediated vasodilatation, leucocyte adhesion to the vascular endothelium and epithelial permeability, all of them involved in the inflammatory responses induced by these cells (Kanwar et al., 1994; Kimura et al., 1999).

3.10.2.2 *Nitric oxide role in neutrophil activation*

Neutrophils are relevant to the non-specific immune response to bacterial infection. The neutrophil action is characterised by adhesion to the vascular endothelium followed by migration into tissues, oxygen radical-dependent killing of microbes and their elimination, together with damaged tissue debris, by phagocytosis. Since the agents released by activated neutrophils are potentially toxic to host tissues, neutrophils are subsequently eliminated by apoptosis and engulfment by macrophages. Thus, modulation of the activation status of neutrophils is of key importance in determining the balance between immune defense and host injury (Armstrong 2001). Human blood neutrophils, stimulated in vitro with monocytes-derived cytokines, and neutrophils from inflamed exudates are reported to express iNOS (Evans et al., 1996; Armstrong 2001). In spite of these reports, there is still controversy as to whether human neutrophils can produce functionally significant levels of NO. For example, Miles et al. (Miles et al., 1995) reported that rat peritoneal neutrophils express iNOS, whereas circulating and extravasated human neutrophils, derived from peritoneal dialysis of peritonitis patients, did not express iNOS mRNA, iNOS protein and enzymatic activity. Rat neutrophils express the constitutive neuronal type NOS RNA and protein. Accordingly, these cells generate basal levels of nitrate/nitrite. Human neutrophils, on the other hand, also express nNOS mRNA, however the nNOS enzyme and NO production were not detected (Greenberg et al., 1998). Overall, it appears that there are differences between human and rodent neutrophils in terms of NO production and, possibly also in the NOS isoforms involved. There is considerable evidence, however, that NO and cyclic GMP act as endogenous mediators of the chemotactic response of neutrophils. NOS inhibitors, in fact, reduced neutrophil chemotaxis induced

by the bacterial peptide n-formyl-methionyl-leucyl-phenylalanine (fMLP) (Wanikiet et al., 1997; Belenky et al., 1993). The role of iNOS-derived NO in neutrophil migration has also been examined in gene knockout studies and differences in kinetics were observed between the two types of mice (Ajuebor et al., 1998). Despite results from studies with NOS inhibitors indicating that endogenous NO acts as a mediator of neutrophil chemotaxis, paradoxically NO donors inhibit various aspects of neutrophil activation, including chemotaxis (Wanikiet et al., 1997). An explanation for these contradictory findings has been proposed by R. Armstrong (Armstrong 2001): NO effects are concentration-dependent, whereby low concentrations stimulate chemotaxis and high concentrations are inhibitory. Thus, the high increase in NO and cyclic GMP observed concomitant with neutrophil activation might represent a negative feedback pathway.

Phagocytosis is a relevant function of neutrophils, however evidence for a role of NO in this process is still limited. An early study suggested that endogenous NO could play a role in human neutrophil phagocytosis since anucleate neutrophil cytoplasts decreased their uptake and killing of *staphylococci* in the presence of the NOS inhibitor L-NMMA, an effect that was reversed by L-arginine (Malawista et al., 1992). Subsequently, L-arginine supplementation was shown to increase phagocytosis of *staphilococcus* by human neutrophils (Moffat et al., 1996). In contrast to these findings, the NO donor GEA 5171 has been shown to inhibit rather than stimulate neutrophil phagocytosis (Forslund et al., 1997). However, no studies with other NO donors have been carried out yet.

3.10.2.3 *Natural killer cells and Nitric oxide*

Natural killer (NK) cells, a major component of innate immunity, play an important role in host resistance to infections from bacteria, viruses and parasites (Biron 1997; Scharon-Kersten et al., 1997). In addition, NK cells are involved in immune surveillance of tumors and rejection of transplanted organs (Whiteside et al., 1995; Lanier 1995) and they can also influence the adaptive immune system and direct the pattern of T responses (Kos 1998). The functions of NK cells, as well as their maturation and differentiation, are regulated by various stimuli, including IL-2, IL-12, IL-15, and IL-18. In particular, unlike most T lymphocytes, resting NK cells express functional IL-2 receptor $\beta\gamma$ (IL-2R $\beta\gamma$) and respond to IL-2 without prior stimulation. Once activated by IL-2, NK cells acquired enhanced cytotoxic function (known as lymphokine-activated killing, LAK) recognizing a broader range of targets and killing with increased lytic potential (Cifone et al., 2001).

Natural killer cells employ cytolysis and cytokine production as major effector mechanisms. The final outcome of their activity results from a balance between activation of stimulatory and inhibitory receptors and ligands. Natural killer cells express a number of inhibitory receptors that recognize MHC class I molecules expressed on normal cells (Cifone et al., 2001). Insufficient expression of class I molecules, or lack of expression of one or more class I alleles render target cells more prone to NK-mediated lysis, as originally proposed by Ljunggren and Karre (Ljunggren et al., 1990) in the “missing-self hypothesis”. Thus, cells that lack class I molecules as a consequence of infection or mutations are sensitive to cytolysis by NK cells. Some tumor cells expressing high levels of autologous class I molecules are nevertheless sensitive to cytolysis by NK cells, indicating that these molecules are not the only

mechanism of target cell discrimination by NK cells (Moretta et al., 1996). Many receptors responsible for NK cell triggering in the process of natural cytotoxicity (natural cytotoxicity receptors, NCR) have been characterized (Moretta et al., 2000). Upon cross-linking, NCR mediate NK cell triggering, leading to target cell lysis and cytokine production. The function of NCR is normally downregulated by co-aggregation with killer inhibitory receptors. Thus, induction of NK cell activation via NCR occurs in the absence of a killer inhibitory receptor-HLA class I interaction (Cifone et al., 2001).

Natural killer-mediated cytotoxicity is directly dependent upon concentration of L-arginine in the culture medium (Cifone et al., 1994) and L-NMMA, a non-specific inhibitor of NOS, abolishes, in a dose dependent fashion, the cytotoxic activity of NK cells (Reif et al., 1995). This evidence supports a role for NO in the lytic events mediated by NK. Nitric oxide is also an effector molecule that mediate, at least partially, the tumoricidal effects of rat IL-2-activated NK cells, as it occurred with fresh NK cells (Yim et al., 1995; Jyothi et al., 1999). On the other hand, increasing levels of NOS activity were observed during LAK generation, a process found to be arginine-dependent (Cifone et al., 1994). The role of NO in LAK generation has been confirmed by Juretic et al. (Juretic et al., 1994), who reported that L-NMMA is inhibitory in the mouse. In human LAK cells, however, no dependence on NO could be demonstrated, suggesting a different regulation of the NO pathway in the two species. The analysis of iNOS expression at mRNA and protein levels in IL-2-treated NK cells established that NO generation is due to the induction of iNOS, rather than eNOS or nNOS (Cifone et al., 1999). Consistent with these findings, mice genetically lacking iNOS have impaired NK cell killing of YAC-1 target cells (Diefenbach et al., 1998). Nitric oxide affects NK

cells expression and production of cytokines such as IFN- γ or TGF- β (Bogdan et al., 2000). The activation of iNOS/NO is necessary for the activation of Tyk2 kinase and thus for IL-12 and IFN- α/β signalling in NK cells (Diefenbach et al., 1999).

3.10.2.4 *Nitric oxide and T cell immunity*

In rodent mixed lymphocyte cultures and in vivo models of T cell-mediated immunity macrophage-derived NO was first shown to inhibit T lymphocyte proliferation (Langrehr et al., 1993; van der Veen 2001). Subsequent studies in mice suggested that NO, preferentially produced by the Th1 subset of T helper lymphocytes (Taylor-Robinson et al., 1994), inhibits selectively Th1 responses and the production of the Th1 cytokine, IFN- γ (Wei et al., 1995). These findings led to the speculation that Th1 cell-derived NO could down-regulate Th1 cell responses in an autocrine manner, and at the same time promote Th2 responses, possibly leading to enhanced production of IgE and promotion of IgE-mediated diseases such as asthma (Barnes et al., 1995). However, these findings and conclusions have since been questioned (van der Veen 2001; Thuring et al., 1995; van der Veen et al., 1999; Bauer et al., 1997). For example, Thuring et al. (Thuring et al., 1995) found no evidence of NO production by mouse lymphocytes or T cell clones. Likewise, van der Veen et al. (van der Veen et al., 1999; van der Veen 2001) found that mouse Th1 cells do not express NOS-2 and also that NO inhibits equally well the proliferation of both mouse Th1 and Th2 cells, without inhibiting production of IFN- γ and other cytokines. Another study found that NO inhibits production of both Th1 and Th2 cytokines by human T lymphocytes (Bauer et al., 1997).

There is evidence that NO has different effects on Th1 differentiation depending on its concentration. Indeed, Th1 cells produce IFN- γ which activates macrophages to express high levels of iNOS, and produce large amounts of NO that inhibit the expansion of Th1 cells by a feedback mechanism (Huang et al., 1998). Low concentrations of NO, on the other hand, have a selective enhancing effect on the induction and differentiation of Th1, but not Th2 cells. This bi-phasic function of NO in immune regulation may be important for the fine tuning of the immune system (Niedbala et al., 1999).

Because of its capacity to induce apoptosis (Brune et al., 1999), NO might play a role as effector molecule in the selection and development of T cells in the thymus. In mouse, rat or human thymocytes, iNOS protein is absent (Tai et al., 1997; Aiello et al., 2000; Mouliau et al., 2001). In the murine thymus, however, very few iNOS⁺ cells were detected in unstimulated animals, and a major increase in the number of iNOS⁺ cells was observed in both fetal and adult thymuses after stimulation with an anti-CD3 antibody (Tai et al., 1997). In the human thymus, on the other hand, iNOS⁺ cells are present in the cortex, in the medulla and around the vessels, and some of them are epithelial. Consistent with this result cultured thymic epithelial cells produce iNOS when activated by IFN- γ , TNF α and IL1 β , 3 cytokines potentially produced by the thymic microenvironment (Mouliau et al., 2001). TCR-activated double-positive thymocytes are highly sensitive to the killing by NO (in particular by peroxynitrite), whereas single-positive thymocytes remain viable upon exposure to NO (Tai et al., 1997; Aiello et al., 2000; Mouliau et al., 2001; Fehsel et al., 1995; Brito et al., 1999). These data suggest that NO released by iNOS-positive thymic stromal cells is one of the

factors mediating deletion of double-positive thymocytes. The function of eNOS expression in thymocytes is still unknown (Cruz et al., 1998).

3.10.2.5 Nitric oxide and dendritic cells: a fascinating story still at its beginning.

Macrophages, which may share a common ontogeny with DCs, respond to stress (such as LPS or inflammatory cytokines) with the generation of NO (Stuehr et al., 1987). Such a response was never reported in DCs stimulated by cytokine or LPS until 1996, when Thomson described the production of NO by highly purified DEC 205⁺ DCs propagated from mouse bone marrow by granulocyte/macrophage-colony stimulating factor (GM-CSF) plus interleukin-4 (IL-4). Nitric oxide synthesis was found to be induced in DCs by IFN- γ and LPS, and blocked by the NOS inhibitor N^G-monomethyl-L-arginine (NMMA) (Bonham et al., 1996). Induction of iNOS mRNA was demonstrated in purified murine DCs stimulated with LPS and IFN- γ , and the intracellular protein product was visualized by two-color flow cytometry. Of particular interest was the observation that only a subpopulation of highly purified IFN- γ plus LPS-stimulated DCs express iNOS. Nitric oxide was also detected in the supernatants of primary mixed leukocytes cultures containing high concentrations of DCs with respect to purified allogeneic T cells. Furthermore, inhibition of NO release in these cultures by NMMA resulted in an increase in T cell proliferation. These observations suggest that NO may be an important soluble mediator of the interaction between DCs and activated T cells.

T lymphocytes, moreover, can play key roles in the regulation of NOS expression since CD40 ligation by mAb does induce NO production by murine DCs. In

addition to its ability to inhibit T cell proliferation, NO was also shown to induce programmed cell death in DCs (Lu et al., 1996; Bonham et al., 1996). Experiments in rodents show in fact that generation of NO by iNOS-expressing thymic DCs contributes to the clonal deletion of thymocytes (Aiello et al., 2000). Spontaneous remission of experimental allergic encephalomyelitis (EAE), a mouse model for multiple sclerosis, is usually associated with prominent apoptosis. Recovery from EAE is associated with functional changes in DC functions, including elevated proliferation, increased IFN- γ secretion and NO production. Since DCs-derived NO results in apoptosis of autoreactive CD4⁺ T cells, thereby inhibiting autoreactive T cell responses, such a NO negative feedback loop may contribute to remission of EAE (Downing et al., 1998; Xiao et al., 1999).

So far, regulation by NO of human DC function has been poorly investigated. At variance with rodents, iNOS expression and NO production by human-derived DCs have not been reported, except in defined pathological condition, namely primary biliary cirrhosis, chronic hepatitis B and hepatocellular carcinoma. Dendritic cells from patients affected by these diseases produce NO, and their high endocytic ability reveals an immature phenotype, suggestive of a defective maturation programme (Wang et al., 2001; Yamamoto et al., 1998; Ninomiya et al., 1999). Dendritic cells from these patients express significantly lower levels of costimulatory molecules and HLA DR; furthermore their T cell stimulatory activity is lower than in normal DCs.

I can conclude that the existence of a role of NO in regulating the function of human DCs, although supported by extensive information, appears still poorly understood. The scope of my thesis' work has been to initiate the filling of this gap.

4 AIMS OF THE WORK

The goal of this thesis was to evaluate if NO can indeed influence human DC functions when the latter cells are exposed to maturation stimuli, and to establish the molecular mechanisms of this NO action.

Specific aims were:

1. To establish whether NO can modulate DC endocytosis of soluble antigens.
2. To establish whether exposure of DCs to NO does influence their ability to induce T lymphocyte activation.
3. To establish whether NO does influence expression of membrane molecules that are upregulated during DC maturation.
4. To identify DC-produced factors modulated by NO which are involved in T cells activation.

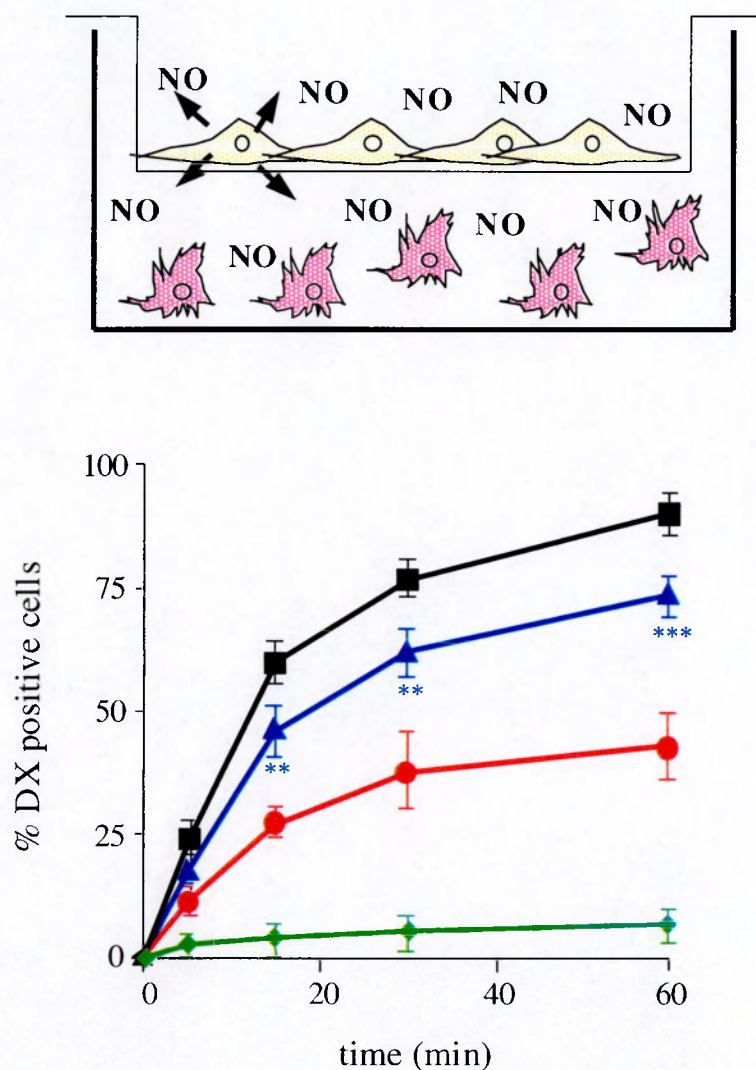
5 RESULTS

5.1 Nitric oxide reverses the inhibition of endocytosis induced by tumour necrosis factor- α in dendritic cells

5.1.1 Nitric oxide modulates endocytosis in dendritic cells undergoing TNF- α -induced maturation

Human monocyte-derived DCs retain their ability to uptake antigens. This ability is progressively lost when DCs are treated with TNF- α (Sallusto et al., 1994). I have investigated the role of NO in the modulation of the endocytic activity of DCs using the well characterized fluorochrome-labelled FITC-dextran, as a marker of endocytosis. Two different sources of the gaseous messenger were used: the NO donors DETA-NO and SNAP; and the N9 murine microglial cells activated with polystyrene microspheres and IFN- γ , a treatment which induces expression of iNOS and continuous generation of bioactive NO (Sciorati et al., 1999; Corradin et al., 1993). In the first experimental approach, immature DCs were incubated in a double chamber system with or without TNF- α (200 ng/ml, 48 h), in the presence or absence of activated N9 cells. Dendritic cells were then resuspended in cytokine-free medium and incubated with FITC-dextran, and internalisation of the latter measured by flow cytometry. In the untreated, immature DCs incubated at 37°C, uptake of FITC-dextran was found to proceed linearly for 15 min and then to progressively level off. In contrast, at 4°C no uptake was detected (Fig. 9). In DCs pretreated with TNF- α for 48 h significantly decreased endocytosis of FITC-dextran was found at all time-points of the experiment, i.e. up to 1 h (Fig. 9).

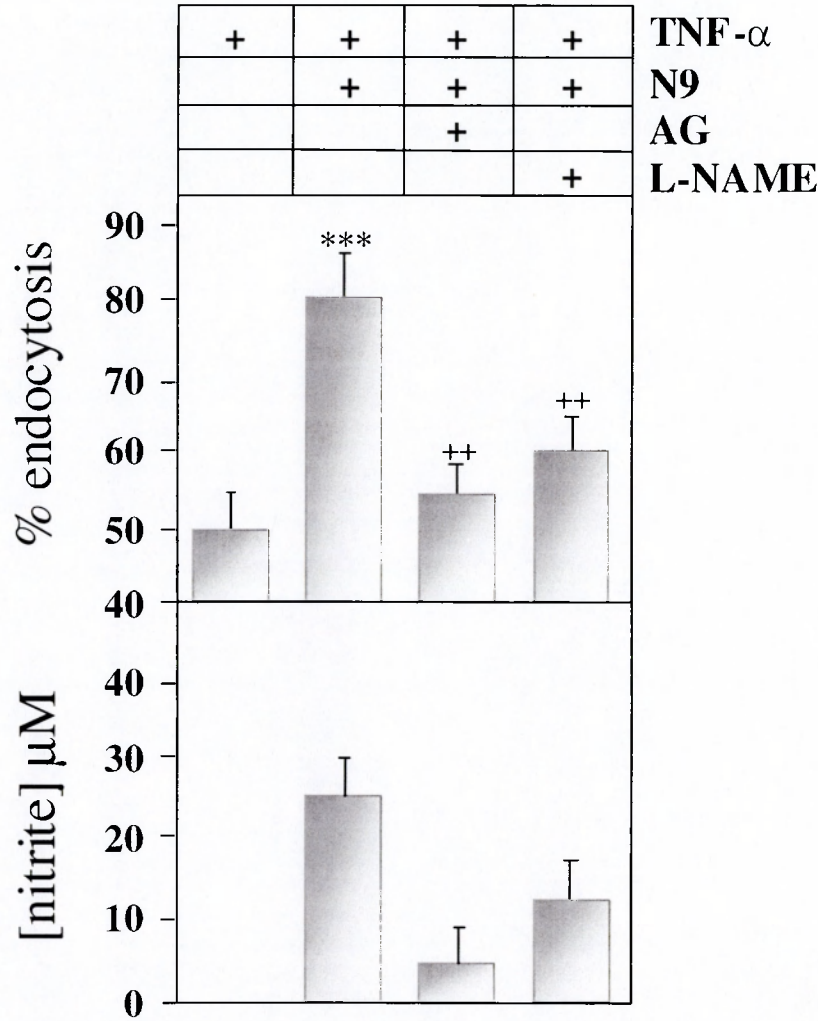
Figure 9 Effects of NO generated by iNOS-expressing N9 cells on the endocytic activity of DCs treated with TNF- α



Immature DCs were cultured without TNF- α (■), with TNF- α (●), or with TNF- α and iNOS-expressing N9 cells (▲) for 48 h. Cells were then washed, suspended in fresh culture medium with or without FITC-dextran (1 mg/ml) and endocytosis at 37°C analysed at the indicated time-points as described in the Methods. As a control, endocytosis of FITC-dextran was measured also at 4°C in cells which were not exposed to TNF- α (◆). Endocytosis was calculated as % of cells positive to FITC-dextran (DX) with respect to cells treated in the same way but not exposed to the fluorescent dye. Statistical probability vs cells treated with TNF- α alone is indicated by the marks * and calculated as described in the Methods ($n = 5$).

This effect of TNF- α was not due to cytotoxicity since cell viability was 94 ± 3.8 % after the 48 h pretreatment with the cytokine ($n = 5$). When the preincubation with TNF- α was carried out in the presence of activated N9 cells the inhibition of endocytosis was largely prevented at all time-points investigated (Fig. 9). This effect was suppressed when incubation of DCs with activated N9 cells was carried out in the presence of the NOS inhibitors aminoguanidine (1 mM) or L-NAME (1 mM) (Fig. 10). Moreover, the effect of the inhibitors was proportional to their ability to reduce NO release by the N9 cells, measured as nitrite accumulation in the culture medium (Fig. 10, lower panel).

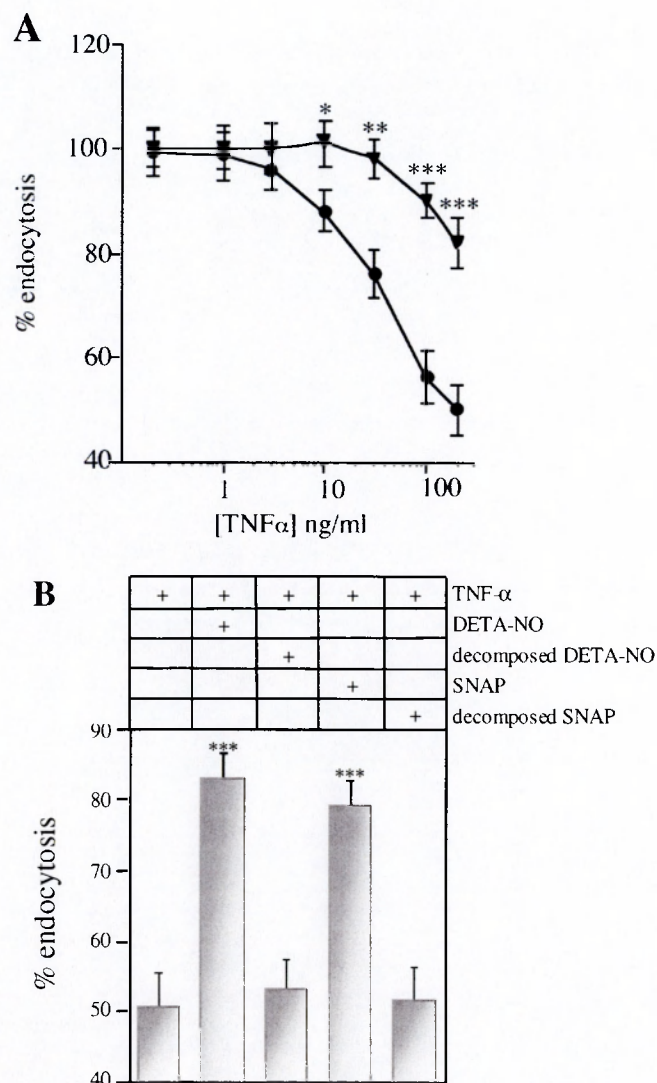
Figure 10 NOS inhibitors abolish the effect of N9 cells on endocytosis activity of DCs treated with TNF- α



Immature DCs were incubated for 48 h with or without TNF- α (200 ng/ml), in the presence or absence of iNOS expressing N9 cells, the NOS inhibitors aminoguanidine (AG, 1 mM) and L-NAME (1 mM), as indicated in the key. The incubation medium was removed and the nitrite concentration in it measured as described in the Methods. Cells were resuspended in fresh culture medium with or without FITC-dextran for 30 min. Endocytosis was calculated as described in A and values expressed as % of those measured in cells incubated without TNF- α (100 %). In both panels statistical probability vs cells treated with TNF- α alone is indicated by the marks * and calculated as described in the Methods. The marks + in panel B refer to the statistical probability vs DCs treated with TNF- α in the presence of N9 cells ($n = 5$).

In the second experimental approach, immature DCs were incubated for 48 h with TNF- α (0.2-200 ng/ml) in the presence or absence of either DETA-NO (100 μ M) or SNAP (200 μ M). Diethylenetriamine-nitric oxide adduct (DETA/NO) is a long-acting nucleophile molecule developed for the controlled release of NO (Wink DA and Kasprzak KS, 1991, Science). At the concentration used, this NO donor releases a constant flux of 50 ± 2.3 nM NO ($n = 4$), as measured by a NO electrode (Clementi et al., 1998). Also in this experiment TNF- α induced a concentration-dependent inhibition of endocytosis (Fig. 11 A) which was largely prevented by either NO donor (Fig 11, A and B). Decomposed DETA-NO and SNAP, which are unable to release NO as measured by a NO-sensitive electrode, did not have any significant effect on the TNF- α inhibition of endocytosis (Fig. 11 B). Moreover, NO donors or activated N9 cells failed to induce any significant effect on endocytosis of DCs not pretreated with TNF- α (not shown). Moreover, NO donors did not induce cytotoxicity (viability vs untreated controls was 95 ± 5.0 % and 93 ± 4.3 % after the 48 h incubation with DETA-NO and SNAP, respectively, $n = 3$).

Figure 11 Effects of NO generated by NO donors on the endocytic activity of DCs treated with TNF- α



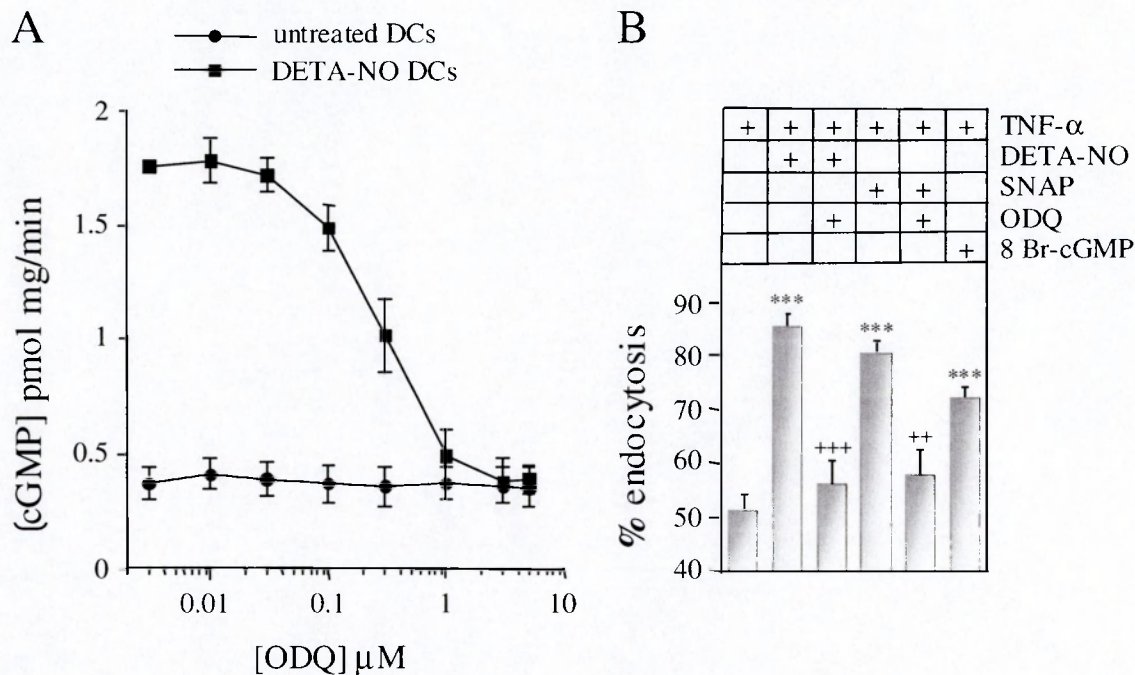
A: Immature DCs were incubated for 48 h with or without TNF- α (0.2-200 ng/ml) in the presence (triangles) or absence (circles) of DETA-NO (100 μ M), washed and resuspended in fresh culture medium with or without FITC-dextran.

B: Immature DCs were incubated for 48 h with or without TNF- α (200 ng/ml) in the presence or absence of DETA-NO (100 μ M) or SNAP (200 μ M) either able or unable to release NO (decayed compounds) as indicated in the key. In both panels endocytosis was calculated as in Fig. 5 after 30 min incubation with or without FITC-dextran. Statistical probability vs cells treated with TNF- α alone is indicated by the marks * and calculated as described in the Methods ($n = 5$).

5.1.2 The effect of nitric oxide on endocytosis of dendritic cells undergoing TNF- α -induced maturation is cGMP-dependent

Nitric oxide biological effects are mediated through signalling pathways both dependent and independent of the gas ability to activate guanylate cyclase and generate cGMP (Moncada et al., 1995). Thus I investigated if NO donors could activate guanylate cyclase and increase cGMP generation in immature DCs with respect to untreated controls. In the present experimental setting administration of DETA-NO induced generation of cGMP, which was inhibited in a concentration-dependent way by ODQ, a specific guanylate cyclase inhibitor (Garthwaite et al., 1995) (Fig 12A). The dependence on cGMP of the effects of NO on endocytosis was then investigated. Immature DCs were treated with TNF- α (48 h) in the presence or absence of NO donors, either alone or in the presence of ODQ, or of the membrane-permeant cGMP analogue 8-Br-cGMP (3 mM). As shown in Fig. 12 B, DETA-NO and SNAP prevented the effect of TNF- α on endocytosis of FITC-dextran. This action of the NO donors was inhibited by ODQ and mimicked by 8-Br-cGMP. These results indicate that NO controls endocytosis regulated by TNF- α through the activation of cGMP-dependent pathway(s).

Figure 12 Effects of cGMP on the endocytic activity of DCs treated with TNF- α



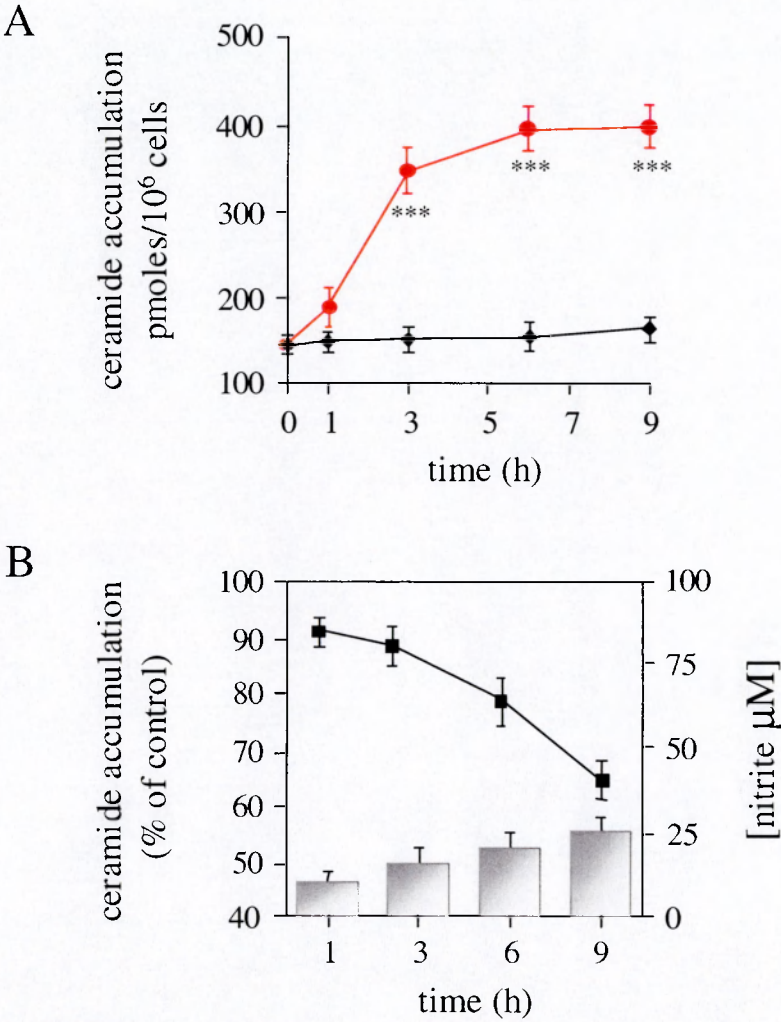
A: Immature DCs were pre-treated for 15 min with the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (0.5 mM), and then incubated for 30 min with increasing concentrations of ODQ (0-5 μ M) in the presence or absence of DETA-NO (100 μ M). cGMP generation in the various experimental conditions was measured using a radioimmunoassay specific for the cyclic nucleotide.

B: Immature DCs were incubated for 48 h with or without TNF- α (200 ng/ml), in the presence or absence of DETA-NO (100 μ M), SNAP (200 μ M), the membrane permeant cGMP analogue 8-Br-cGMP (3 mM), or the inhibitor of soluble guanylyl cyclase ODQ (3 μ M) as indicated in the key. Endocytosis was measured after 30 min of incubation with or without FITC-dextran and calculated as in Fig. 10. Statistical probability vs cells treated with TNF- α alone is indicated by the marks * and calculated as described in the Methods. The marks + refer to the statistical probability in cells treated with TNF- α , ODQ and either NO donor vs cells treated without ODQ ($n = 3$).

5.1.3 Accumulation of ceramide induced by TNF- α in dendritic cells is inhibited by nitric oxide in a cGMP-dependent fashion.

Ceramide, a second messenger generated by the activation of the p55 receptor of TNF- α , has been shown to mediate some of the effects of the cytokine in DCs (Sallusto et al., 1996). The time-course of ceramide accumulation by DCs treated with TNF- α is shown in Figure 13 A. The cytokine induced a progressive accumulation of the lipid messenger, which reached a plateau after about 6 h and remained constant thereafter. No significant ceramide accumulation was observed in cells not exposed to TNF- α (Fig. 13 A). When DCs were incubated with the cytokine in the presence of activated N9 cells, ceramide accumulation was reduced and a negative correlation was found between the amount of NO generated, measured as the concentration of nitrite released in the medium, and the intracellular accumulation of ceramide (Fig. 13 B).

Figure 13 Effects of NO and cGMP on the TNF- α -induced ceramide accumulation in DCs

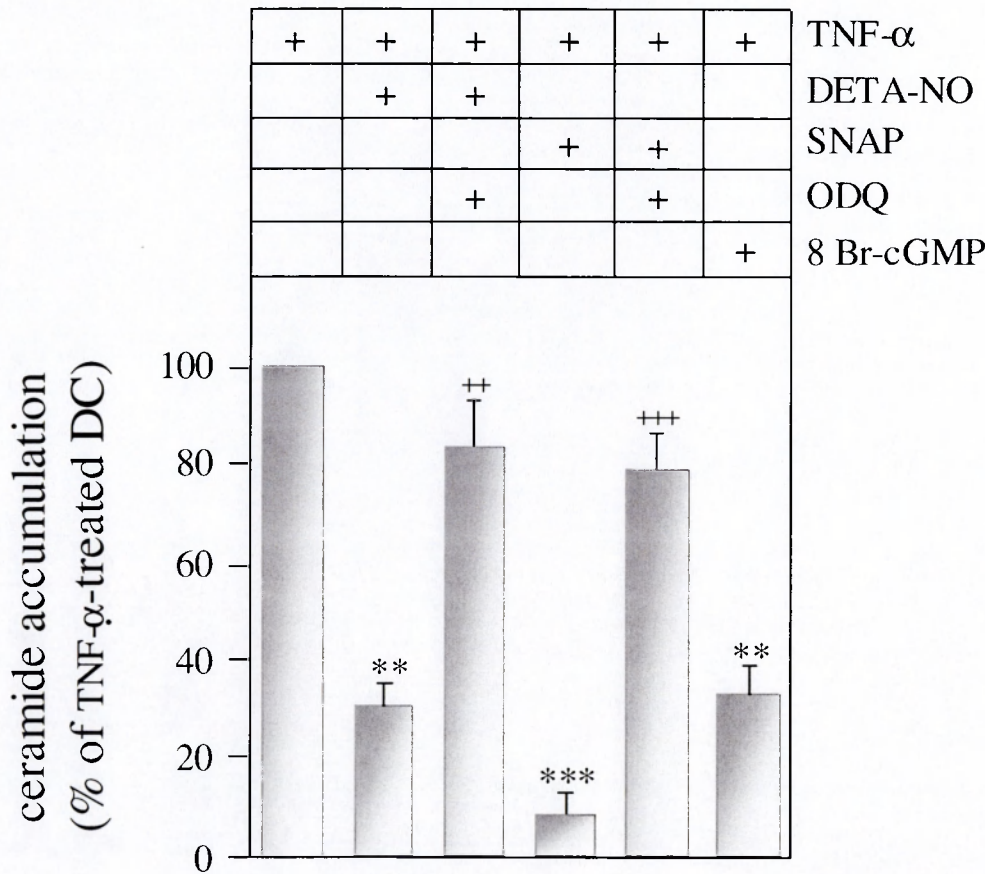


A: immature DCs were treated with (●) or without (◆) TNF- α (200 ng/ml). Cell aliquots were collected at the indicated time-points, lysed and ceramide content measured by thin layer chromatography as described in the Methods. Statistical probability vs cells treated without TNF- α is indicated by the marks * and calculated as described in the Methods.

B: immature DCs were treated with TNF- α (200 ng/ml) and iNOS expressing N9 cells (■) for the indicated time-points. Ceramide accumulation was measured as in A. Values are expressed as % of those measured in DCs treated with TNF- α in the absence of N9 cells (control). The bars show the nitrite concentration in the medium measured as described in the Methods. Statistical probability vs cells treated with TNF- α alone is indicated by the marks * and calculated as described in the Methods ($n = 4$).

A similar inhibition of ceramide accumulation induced by TNF- α was observed in cells incubated with DETA-NO or SNAP (Fig. 14). Also this effect of NO appeared to be cGMP-dependent inasmuch as it was prevented by incubation with the NO donors in the presence of ODQ and was mimicked by 8-Br-cGMP.

Figure 14 The effects of NO on the TNF- α -induced ceramide accumulation in DCs are cGMP dependent.

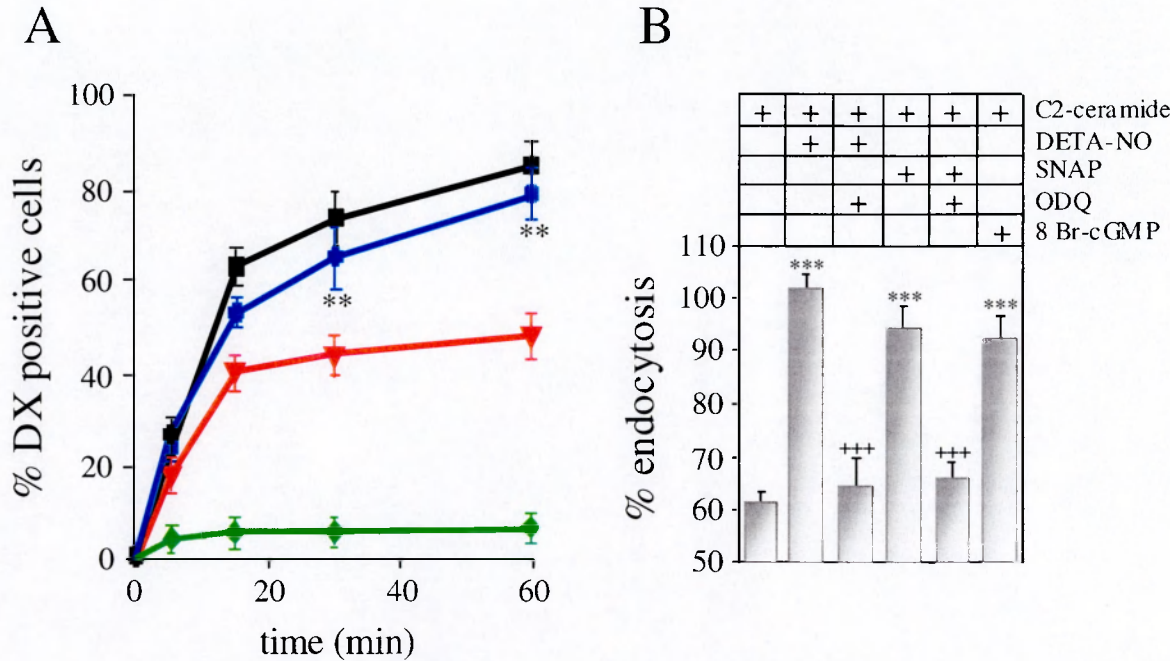


Cells were treated for 6 h with TNF- α with or without DETA-NO (100 μ M), SNAP (200 μ M), 8 Br-cGMP (3 mM) and ODQ (3 μ M) as indicated in the key. Values are expressed as % of those measured in DCs treated with TNF- α . Statistical probability vs cells treated with TNF- α alone is indicated by the marks * and calculated as described in the Methods. The marks + refer to the statistical probability in cells treated with TNF- α , ODQ and either NO donor vs cells treated without ODQ ($n = 4$).

5.1.4 The nitric oxide/cGMP action on dendritic cells endocytosis is exerted both upstream and downstream of the TNF- α -induced generation of ceramide.

Long-term incubation of DCs with exogenous C₂ ceramide (80 μ M, 48 h) resulted in a statistically significant, persistent inhibition of FITC-dextran endocytosis with respect to that observed in untreated, control cells (Fig. 15 A). When DC incubation with exogenous C₂ ceramide was carried out in the presence of DETA-NO (Fig. 10, A and B) or SNAP (Fig. 15 B), the inhibition of endocytosis by the lipid metabolite was reduced. This effect of the NO donors was prevented by incubation with ODQ and mimicked by 8-Br-cGMP (Fig. 15 B). These results indicate that the action of NO is exerted not only via the inhibition of the TNF- α -induced ceramide generation, but also via (a) mechanism(s) active downstream of the latter process. Both these effects of NO appear dependent on cGMP generation.

Figure 15 Effects of NO on the endocytic activity of DCs treated with ceramide



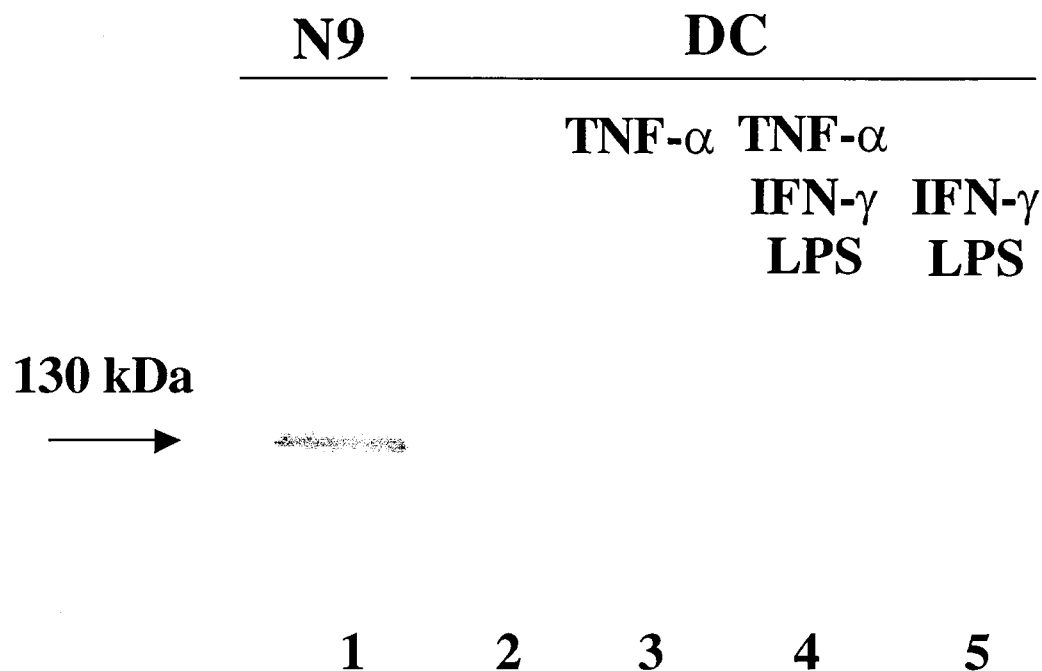
A: immature DCs were incubated without C₂ ceramide (■), with C₂-ceramide (80 μM; ▼), or with C₂-ceramide and the NO donor DETA-NO (100 μM; ◆) for 48 h. Cells were then washed, suspended in fresh culture medium with or without FITC-dextran (1 mg/ml) and endocytosis at 37°C analysed at the indicated time-points as described in the Methods. As a control, endocytosis of FITC-dextran was measured also at 4°C in cells which were not exposed to C₂ ceramide (◇). Endocytosis was calculated as described in Fig 4.

B: immature DCs were incubated with or without C₂-ceramide as in A, in the presence or absence of DETA-NO (100 μM), SNAP (200 μM), ODQ (3 μM), or 8 Br-cGMP (3 mM) as indicated in the key. Endocytosis was measured after 30 min of incubation with or without FITC-dextran, calculated as described in A and values expressed as % of those measured in cells incubated without C₂-ceramide (100 %). In both panels statistical probability vs cells treated with C₂-ceramide alone is indicated by the marks * and calculated as described in the Methods. The marks + in panel B refer to the statistical probability in cells treated with C₂-ceramide, ODQ and either NO donor vs cells treated without ODQ (*n* = 4).

5.1.5 Treatment with cytokines and LPS does not induce generation of nitric oxide by dendritic cells.

Expression of iNOS and generation of NO by DCs has been reported in rodents both *in vivo* (Downing et al., 1998) and in purified DCs after treatment with IFN- γ and LPS *in vitro* (Lu et al., 1996; Xiao et al., 1999). I therefore investigated whether exposure of human-derived DCs to cytokines and LPS also induces expression of iNOS and generation of NO. Immature DCs were incubated for 48 h in the presence or absence of human TNF- α , human IFN- γ (100 U/ml) and LPS (10 μ g/ml), alone or combined as detailed in Fig. 16. NO generation was measured in the culture medium as nitrite formation, and the expression of iNOS analysed by Western blotting of DC lysates. Neither iNOS expression in DCs nor nitrite accumulation in the medium were detected before or after any of the treatments applied (Fig. 16 and not shown). It is possible to conclude that DCs are unable to modulate their endocytosis decrease process by endogenous generation of NO.

Figure 16 Effects of cytokines and LPS on iNOS expression in DCs



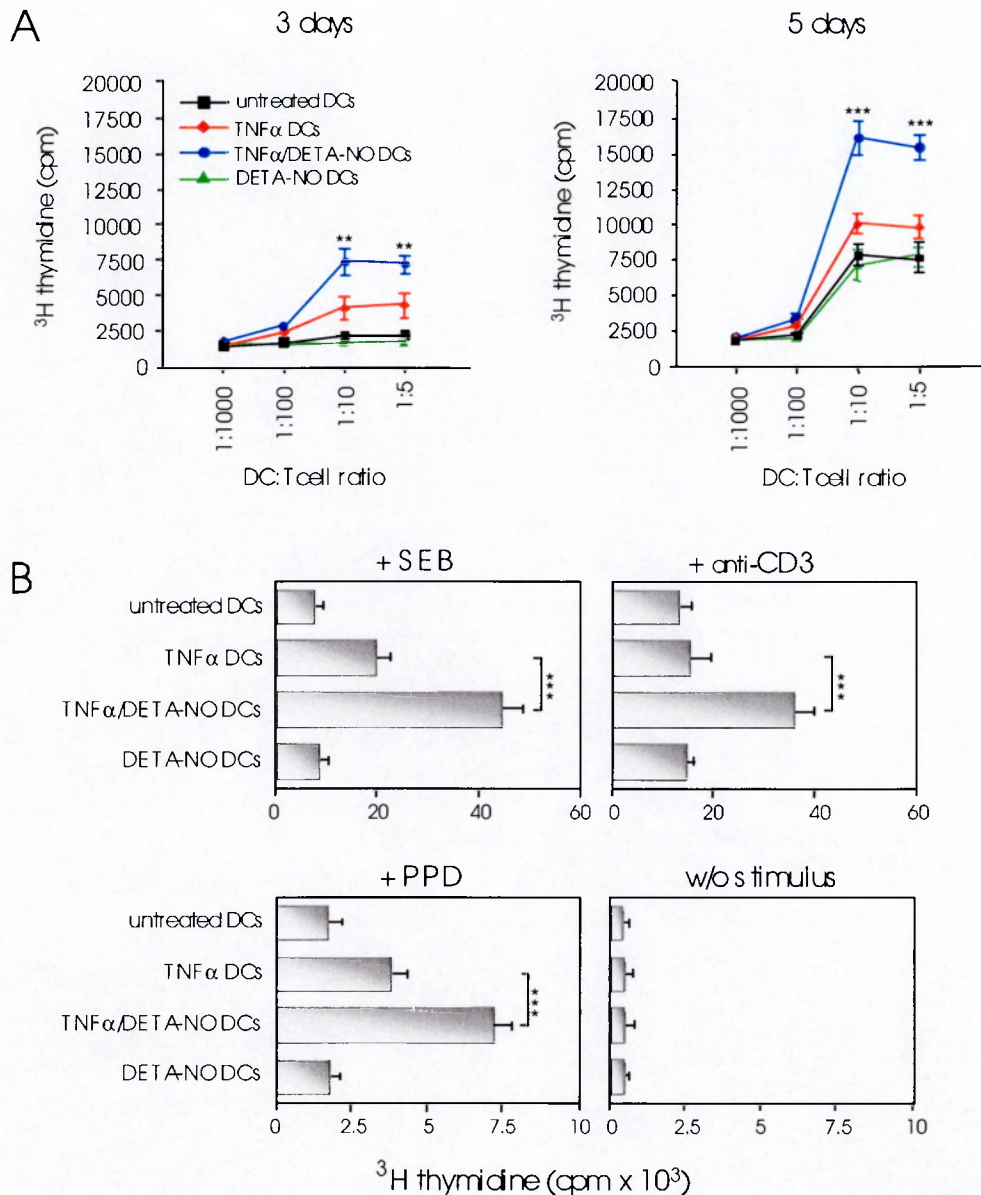
Effects of cytokines and LPS on iNOS expression in DCs. Immature DCs were treated for 48 h in the absence or presence of: TNF- α (200 ng/ml, 2 and 3, respectively); TNF- α , IFN- γ (100 U/ml) and LPS (10 μ g/ml, 4); IFN- γ and LPS (5). Cells were lysed and iNOS expression revealed by Western blotting with an anti iNOS Ab as described in the Methods. As a positive control, N9 cells, activated as described in the Methods, were loaded in parallel (1). The results shown are from one experiment representative of four consistent ones.

5.2 Synergism between nitric oxide and maturation signals in dendritic cells leads to an enhancement of T lymphocyte activation.

5.2.1 T lymphocyte activation by TNF- α -treated dendritic cells is enhanced by nitric oxide.

Immature DCs were treated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of the NO donor DETA-NO (50 μ M), then washed free of the cytokine and the NO donor. As previously reported, this NO donor releases a constant flux of NO and does not affect DC viability. The antigen presenting function of pretreated DCs was evaluated by measuring DC-induced proliferation of either allogeneic T lymphocytes in MLRs (Fig. 17 A), or autologous T lymphocytes stimulated with SEB (0.2 μ g/ml), PPD (1 μ g/ml) or anti-CD3 mAb (0.1 μ g/10⁶ cells) (Fig. 17 B). In all these experimental settings, pre-treatment with DETA-NO alone resulted in no changes in DC-induced T cell proliferation. By contrast, when the NO donor was administered to DCs together with TNF- α , stimulation of T cells was increased. T cell proliferation was significantly higher than that induced by either untreated DCs or DCs pre-treated with TNF- α alone, both in MLRs (Fig. 17 A) and when autologous reactions in the presence of SEB, anti-CD3 mAb or PPD (Fig. 17 B). Regardless of the pre-treatment, DCs alone induced no significant proliferation of unstimulated, autologous T cells (Fig. 17 B).

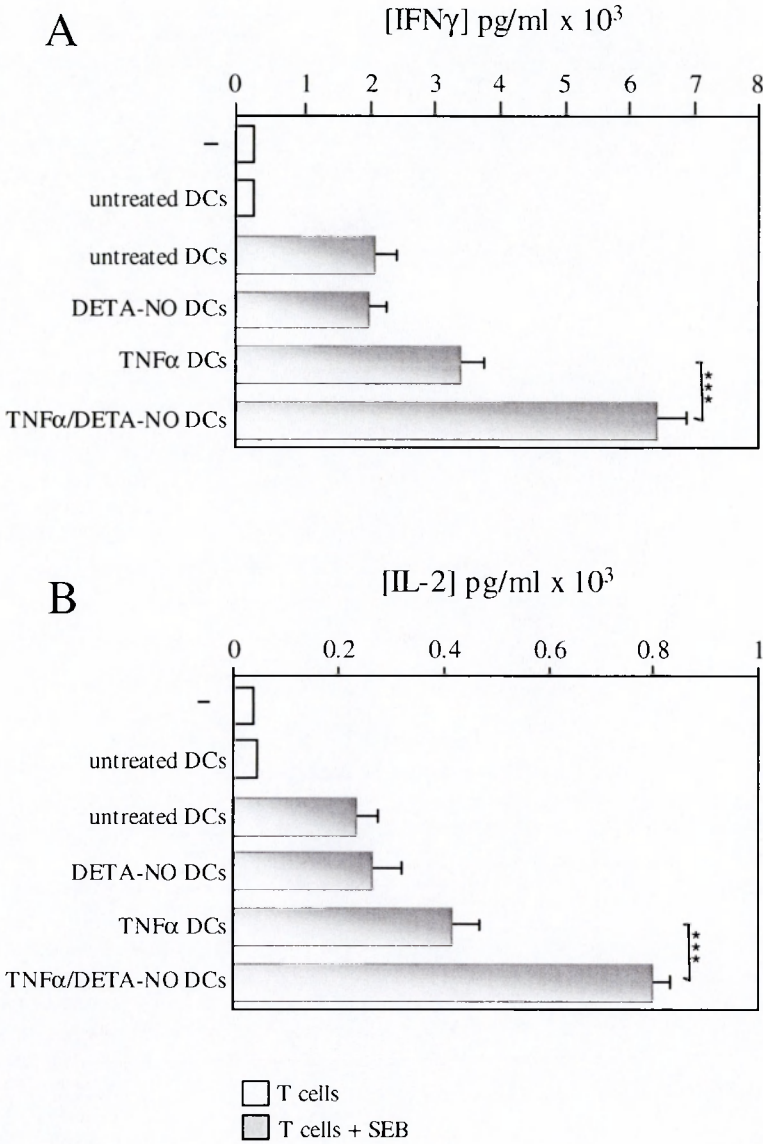
Figure 17 NO enhances the ability of TNF- α -treated DCs to induce T cell activation



Immature DCs were treated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of DETA-NO (50 μM). **A:** These pre-treated DCs were co-cultured at the indicated ratios with a fixed number of allogeneic T cells (10^5 cells/well); proliferation (^3H -thymidine incorporation in T cells) was measured after 3 and 5 days of DC/T cell co-culture. **B:** 2×10^4 pre-treated DCs were co-cultured with 10^5 autologous T cells in the presence of SEB (0.2 $\mu\text{g/ml}$), anti-CD3 (0.1 $\mu\text{g}/10^6$ cells), PPD (1 $\mu\text{g/ml}$) or without any stimulus. Proliferation was measured after 3 days. Asterisks indicate statistical significance, measured as indicated in Materials and methods, of TNF- α /DETA-NO DCs vs. TNF- α -treated DCs ($n = 8$).

The concentration of IFN- γ and IL-2 accumulated in the medium under the various experimental conditions described above was measured. Figure 18 shows the results obtained using SEB-stimulated T cells. IFN- γ and IL-2 released in the presence of DCs pre-treated with DETA-NO alone were similar to those released with immature, untreated DCs. Pre-treatment of DCs with TNF- α resulted in increased release of both cytokines, which was further, significantly enhanced in the presence of the NO donor. In the absence of SEB no significant release of IFN- γ and IL-2 was observed, regardless on whether lymphocytes were cultured alone or in the presence of untreated, immature DCs.

Figure 18 NO enhances the ability of TNF- α -treated DCs to induce IFN γ and IL-2 production by T cells.



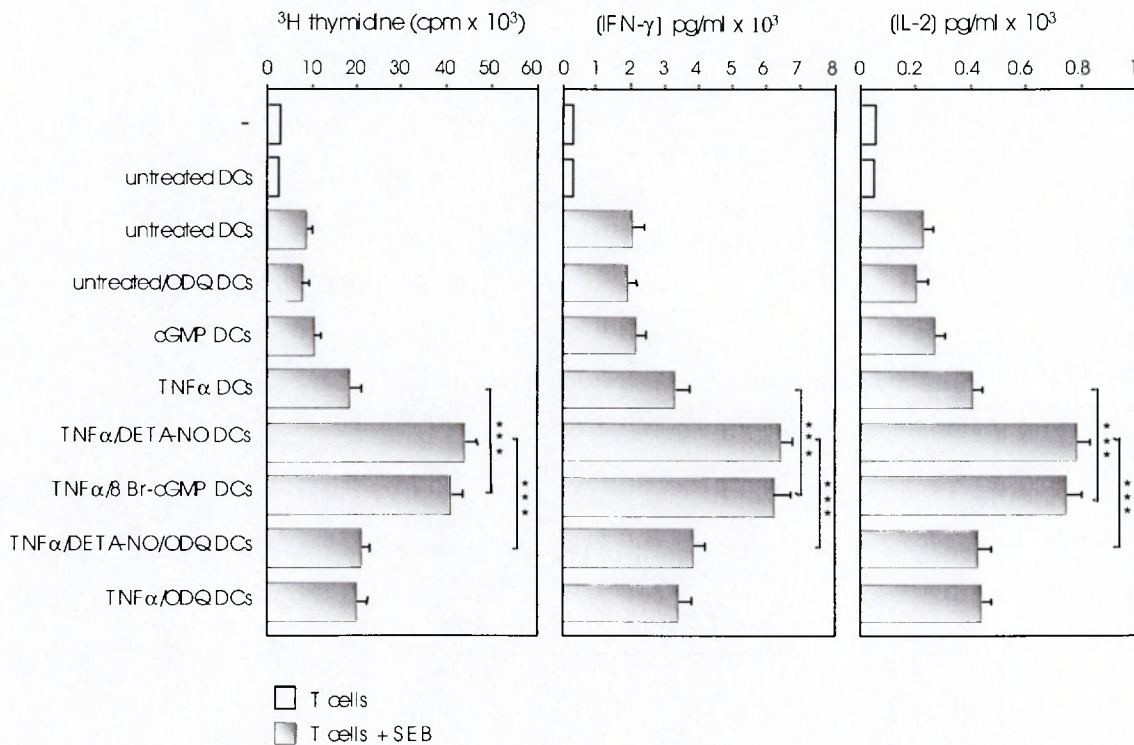
Immature DCs were treated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of DETA-NO (50 μ M). IFN- γ and IL-2 secretion were measured in the supernatant from 3 days co-culture of 10^5 autologous T cells with or without 2×10^4 pre-treated DCs and in the presence or absence of SEB (0.2 μ g/ml). Asterisks indicate statistical significance, measured as indicated in Materials and methods, of TNF- α /DETA-NO DCs vs. TNF- α -treated DCs ($n = 8$).

5.2.2 The effect of nitric oxide on T lymphocyte activation by TNF- α -treated dendritic cells is dependent on generation of cGMP.

Previously I have shown that NO triggers cGMP generation in immature human DCs and that the cyclic nucleotide contributes to regulate endocytosis in these cells. I therefore investigated whether the NO-dependent enhancement of DC antigen presenting ability was mediated by cGMP. To this end, immature DCs were pre-treated with or without TNF- α in the presence or absence of either the membrane-permeant cGMP analogue 8 Br-cGMP, DETA-NO, or DETA-NO together with ODQ. DCs treated with 8 Br-cGMP and TNF- α induced proliferation of T cells and release of IFN- γ and IL-2 higher than those induced by TNF- α -treated DCs, with a pattern similar to that observed using DCs treated with DETA-NO and TNF- α (Fig. 19).

Pre-treatment with ODQ, while ineffective in DCs treated with TNF- α alone, reversed the potentiating effect induced by DETA-NO co-administration with TNF- α on both the proliferation of T cells and the release of cytokines (Fig. 19).

Figure 19 The NO-dependent enhancement of TNF- α -treated DCs to induce T cells activation is mediated by cGMP



Immature DCs were incubated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of DETA-NO (50 μ M), the membrane permeant cGMP analogue 8 Br-cGMP (3 mM), or the inhibitor of soluble guanylate cyclase ODQ (0-5 μ M), as indicated in the keys to the various panels. 10⁵ autologous T cells were then co-cultured with or without 2x10⁴ pre-treated DCs in the presence or absence of SEB (0.2 μ g/ml). T cells activation was evaluated as both proliferation (A) and secretion of IFN- γ and IL-2 (B and C) after 3 days. Asterisks indicate statistical significance, measured as indicated in Materials and methods ($n = 8$).

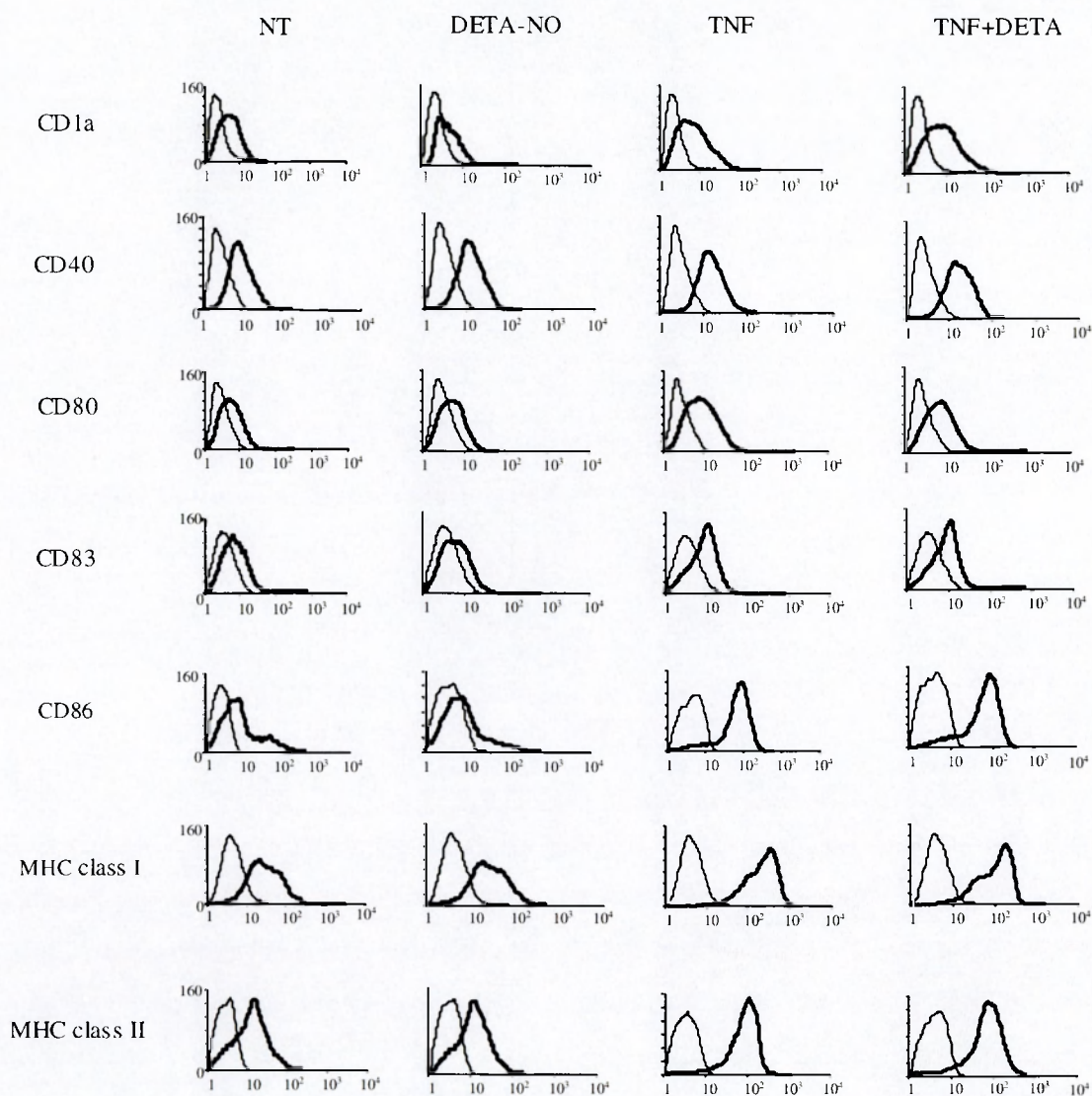
5.2.3 A soluble factor released by dendritic cells treated with TNF- α and nitric oxide is responsible for the enhanced T lymphocyte activation.

TNF- α -induced differentiation of DCs towards a mature, antigen-presenting phenotype involves the co-ordinate up-regulation on the plasma membrane of various proteins, including MHC class I and II and co-stimulatory molecules, as well as the release of soluble factors (Banchereau et al., 2001).

I examined whether NO modulates plasma membrane expression of MHC class I and II, CD1a, CD83 and co-stimulatory CD40, CD80 and CD86 molecules. The pattern of expression of these molecules in both immature and TNF- α -treated DCs is shown in Fig. 20. Treatment of immature DCs for 48 h with DETA-NO alone changed expression of neither MHC class I and II, nor any of the co-stimulatory molecules investigated. Likewise, the NO donor did not modify the pattern of expression of these molecules elicited by a 48 h maturation treatment with TNF- α (Fig. 20).

It is possible to conclude that, at variance with endocytosis, ceramide generation and T cell activation, surface protein finger printing induced by TNF- α in DCs is established independently of the NO regulation.

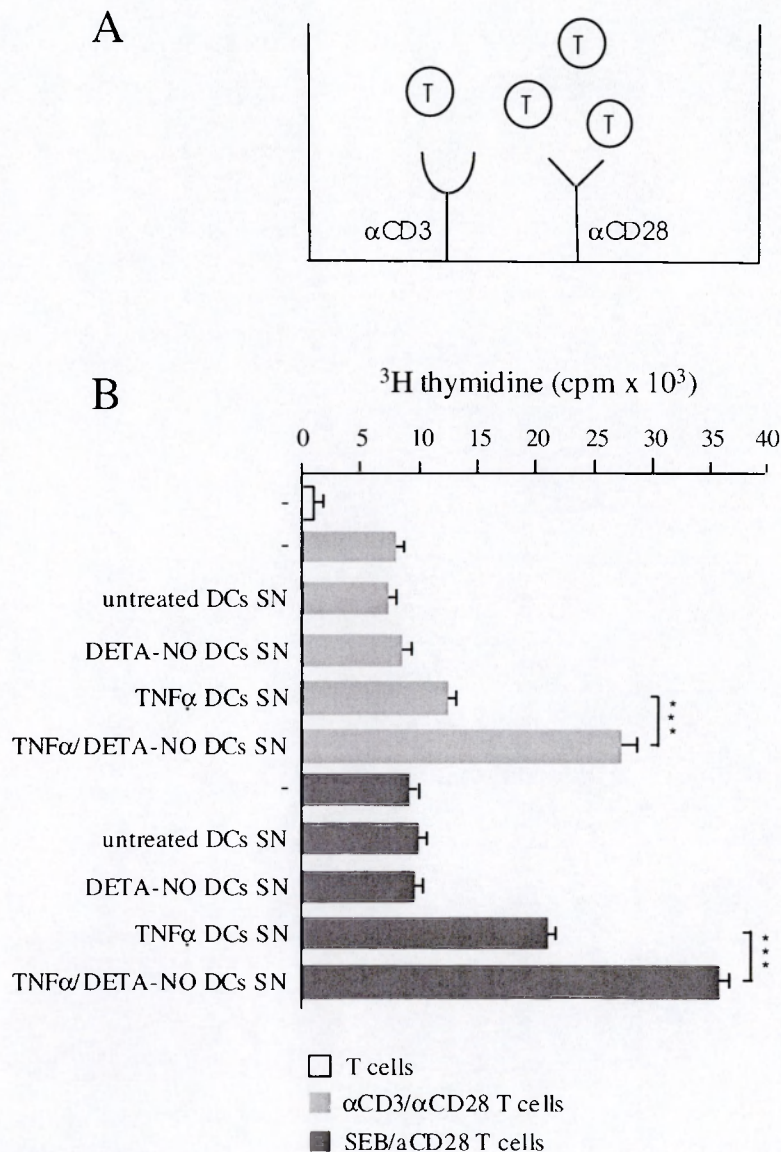
Figure 20 Phenotypic characterisation of DCs induced to mature by TNF- α administered in the presence or absence of NO



Immature DCs were treated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of DETA-NO (50 μ M). Cells preparations were analysed by flow cytometry after staining with FITC-conjugated Abs specific for the surface antigens specified at the top of each columns (solid-line histograms). Cell autofluorescence is indicated by the superimposed, thin-line histograms. The results shown are from one experiment representative of 10 consistent ones.

Whether release of soluble factors accounts for the effects of NO-treated DCs on T cell proliferation was next investigated. Immature DCs were treated for 2 days with or without TNF- α in the presence or absence of DETA-NO, washed free of the cytokine and the NO donor, and maintained for a further 2 days in culture. Culture medium was then removed and assayed for its ability to modulate proliferation of T lymphocytes in an activation system consisting of a CD28-activating mAb administered together with either SEB or anti-CD3 mAb. Both these activation systems triggered significant T cell proliferation responses (Fig. 21). Addition of the supernatant from cultures of either untreated DCs or DCs treated with only DETA-NO did not modify this activation. By contrast, the supernatant from TNF- α -treated DCs increased T cell proliferation (more evidently in the anti-CD28/SEB model). In both the activation systems analysed this increased T cell proliferation effect was significantly greater using supernatants from DCs co-incubated with TNF- α and DETA-NO (Fig. 21).

Figure 21 Release of soluble factors is responsible for the enhanced T lymphocytes activation by DCs pretreated with TNF- α and NO

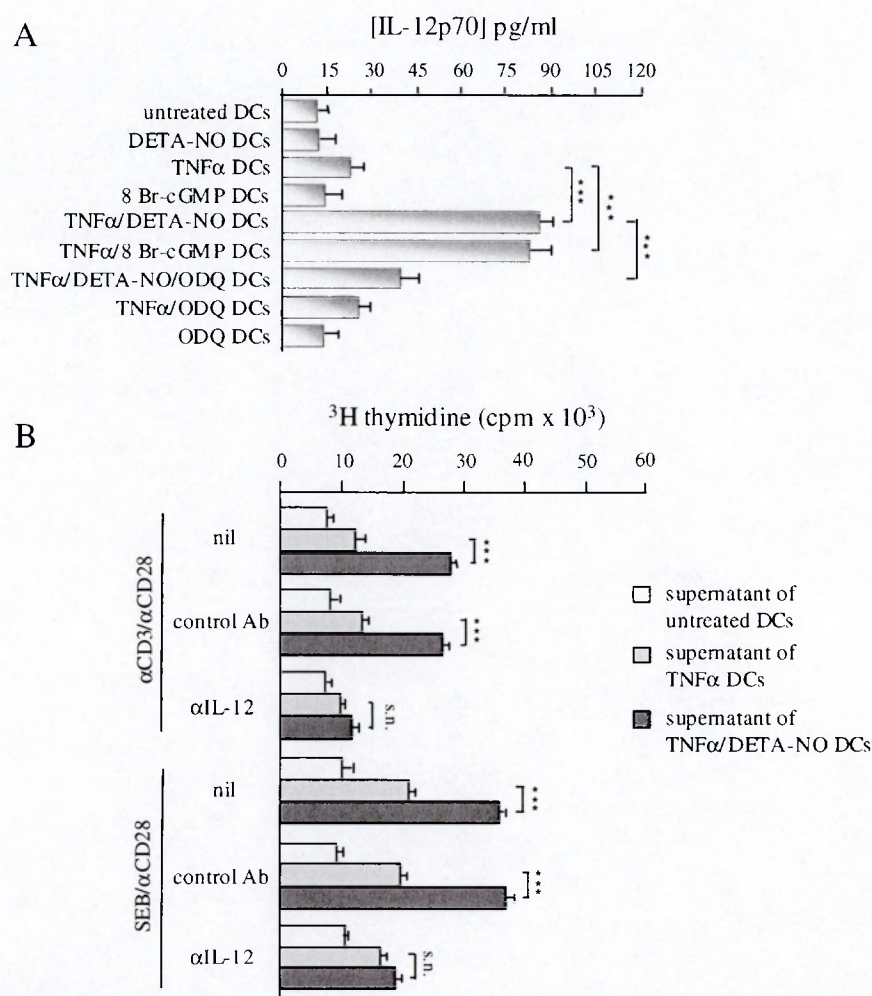


Supernatants were collected from DCs pre-treated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of either DETA-NO (50 μM). T cell proliferation in the presence or absence of the various supernatants, as indicated on the left hand side of panel B, was stimulated for 3 days with either anti-CD3 (1 $\mu\text{g}/\text{ml}$)/anti-CD28 (5 $\mu\text{g}/\text{ml}$) mAbs or SEB (0.2 $\mu\text{g}/\text{ml}$)/anti-CD28 mAb, as shown in the scheme of panel A. Control experiments with unstimulated T cells were run in parallel

5.2.4 Increased release of IL-12 is responsible for the enhanced T cell activation by nitric oxide/TNF- α -treated dendritic cells

Among the pro-inflammatory cytokines released by DCs, IL-12 has been shown to play pivotal roles in T cell activation (Ma et al., 2001). We therefore evaluated whether the stimulatory effect of NO is mediated by release of IL-12. As shown in Figure 22 A, release of the cytokine was slightly increased by pre-treatment of DCs with TNF- α alone and further, significantly enhanced when either DETA-NO or 8Br-cGMP were co-administered with TNF- α . The dependence on cGMP generation of the effect of DETA-NO was further confirmed by the observation that administration of ODQ prevented the co-stimulatory effect of DETA-NO administered with TNF- α , giving rise to IL-12 generation responses similar to those observed in DCs pre-treated with TNF- α alone. Basal IL-12 release was not modified by DC pre-treatment with DETA-NO, 8Br-cGMP or ODQ alone. To evaluate whether this increased secretion of IL-12 was involved in stimulation of T lymphocyte proliferation by DCs, we tested the effect of a neutralising, anti-IL-12 Abs cocktail (Rogge et al., 1997). Inhibition of IL-12 did not modify significantly T cell proliferation induced by the supernatant from both untreated and TNF- α -pre-treated DCs. This applied to both anti-CD28/SEB and anti-CD28/anti-CD3-stimulated T cells (Fig. 22 B). In contrast, the neutralising anti-IL-12 Abs cocktail abolished the enhancing effect on T cell proliferation induced by DETA-NO when combined with TNF- α , giving rise to proliferation values similar to those observed with TNF- α alone. These effects appeared to be specific because they were not observed in the presence of an isotype control Ab.

Figure 22 Release of IL-12 is crucial to enhanced T lymphocyte activation by DCs treated with TNF- α together with NO



Supernatants were collected from DCs pre-treated for 2 days with or without TNF- α (50 ng/ml) in the presence or absence of either DETA-NO (50 μM), 8 Br-cGMP (3 mM) or ODQ (3 μM).

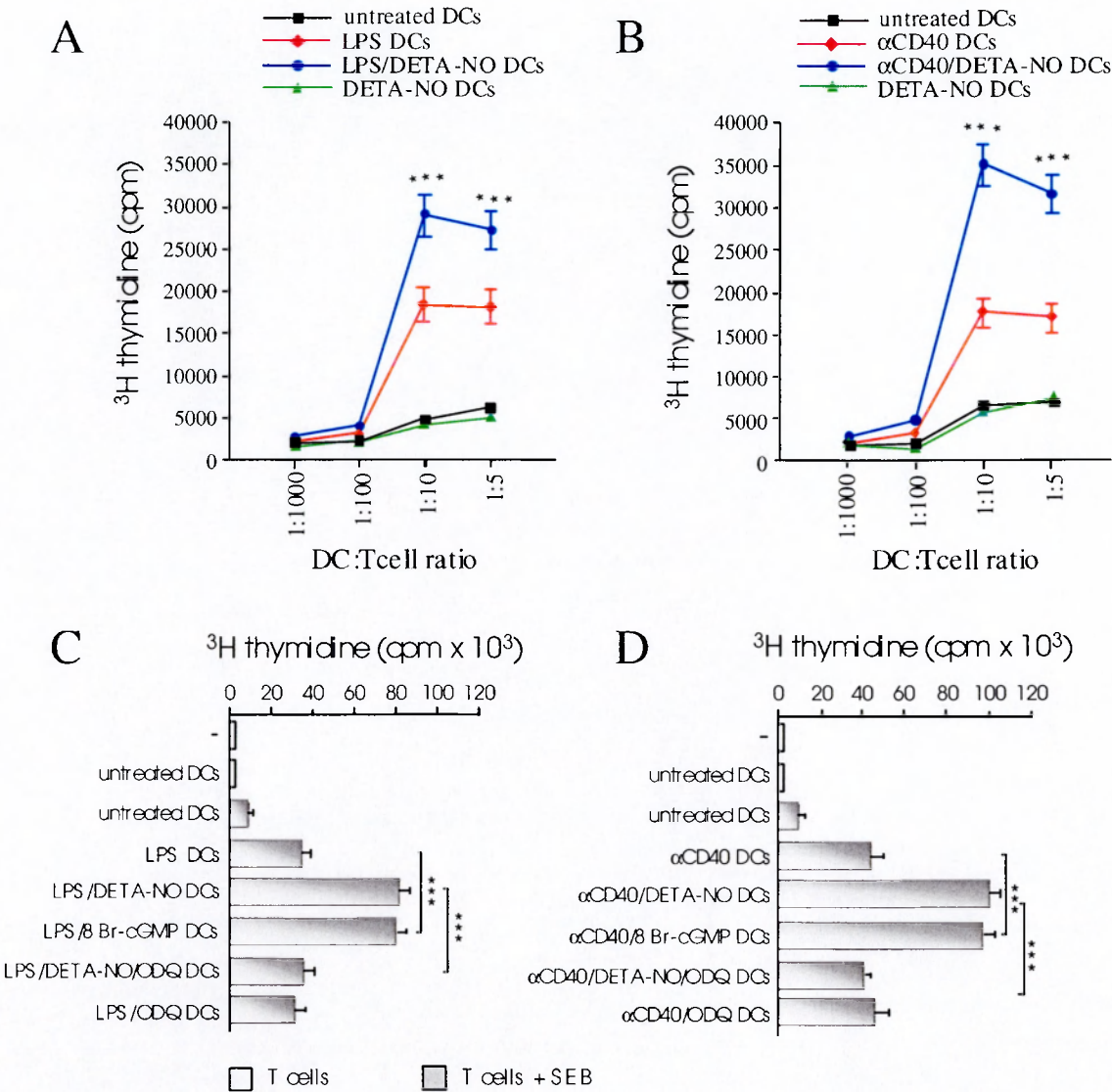
A: concentration of IL-12p70 in the supernatants was evaluated by ELISA.

B: T cell proliferation in the presence or absence of the various supernatants, as indicated on the left hand side, was stimulated for 3 days with either anti-CD3 (1 $\mu\text{g}/\text{ml}$)/anti-CD28 (5 $\mu\text{g}/\text{ml}$) mAbs or SEB (0.2 $\mu\text{g}/\text{ml}$)/anti-CD28 mAb. Control experiments with unstimulated T cells were run in parallel. Experiments were carried out as in the absence (nil) or presence ($\alpha\text{IL-12}$) of an IL-12 neutralising mAb cocktail, as described in the Methods section. Experiments with an isotype control Ab were carried out in parallel. Asterisks indicate statistical significance, measured as indicated in Materials and methods; s.n. statistically not significant ($n = 6$).

5.2.5 Nitric oxide enhances by cGMP-dependent IL-12 release the T lymphocyte activation by dendritic cells matured with either LPS or an anti-CD40 mAb.

To assess whether the effect of NO and the mechanism of its action are specific for TNF- α -induced DC maturation or constitute a more general phenomenon, I investigated DC maturation induced by either LPS or activation of CD40. Immature DCs were treated for 2 days with or without LPS (1 μ g/ml) or an anti-CD40, activating mAb (10 μ g/ml) in the presence or absence of DETA-NO, 8 Br-cGMP, and ODQ, administered in various combinations. The cells were then washed free of all the treatments. When DETA-NO was administered to DCs together with either LPS or the anti-CD40 mAb, the NO donor increased DC induced stimulation of T cells, both in MLRs (Fig. 23, A and B) and in autologous reactions in the presence of SEB (Fig. 23, C and D). Similar to the experiments with TNF- α , in the experiments with LPS- and anti-CD40-treated DCs I found that all the effects of NO were dependent on cGMP generation, since they were mimicked by 8 Br-cGMP and reversed by ODQ (Fig. 23, C and D).

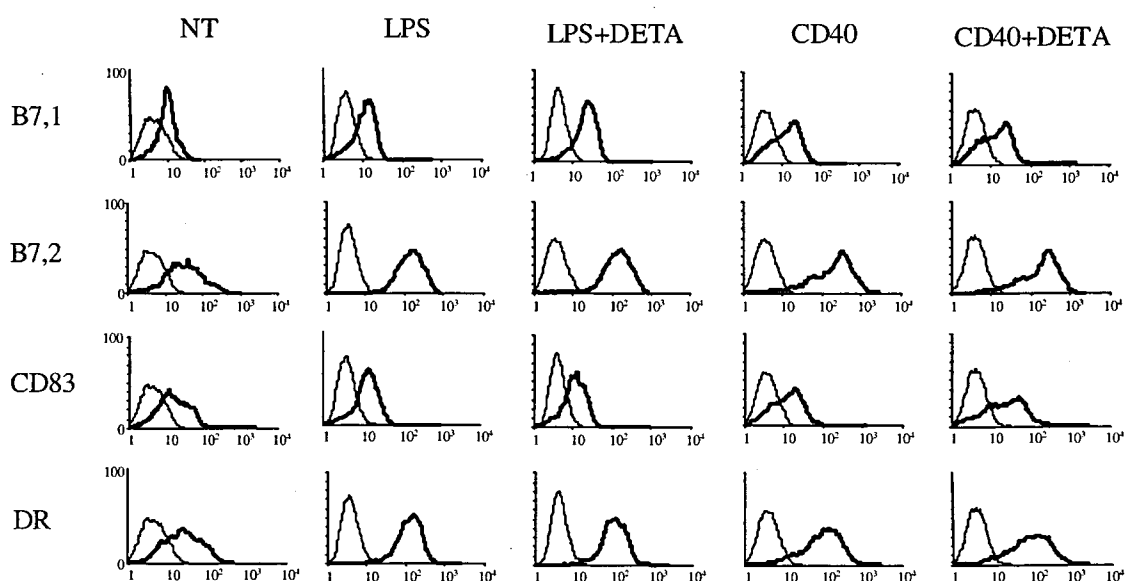
Figure 23 NO enhances the ability of LPS- and anti CD40 mAb-treated DCs to induce T cell activation



Immature DCs were treated for 2 days with or without either LPS (1 μ g/ml, panels A and C) or an anti-CD40 mAb (10 μ g/ml, panels B and D) in the presence or absence of DETA-NO (50 μ M), 8 Br-cGMP (3 mM) or ODQ (3 μ M). (A and B) These pre-treated DCs were co-cultured at the indicated ratios with a fixed number of allogeneic T cells (10^5 cells/well); proliferation (3 H-thymidine incorporation in T cells) was measured after 5 days of DC/T cell co-culture. (C and D) 2×10^4 pre-treated DCs were co-cultured with 10^5 autologous T cells in the presence of SEB (0.2 μ g/ml) or without any stimulus. Proliferation was measured after 3 days. Asterisks indicate statistical significance, measured as indicated in Materials and methods ($n = 6$).

Both LPS treatment and CD40 cross-linking triggered DC maturation, as assessed by increased expression of CD80, CD83, CD86 and MHC class II, and none of which was modified by co-treatment of the cells with DETA-NO (Fig 24).

Figure 24 Phenotypic characterisation of DC maturation induced by LPS or by CD40 cross-linking in the presence or absence of NO

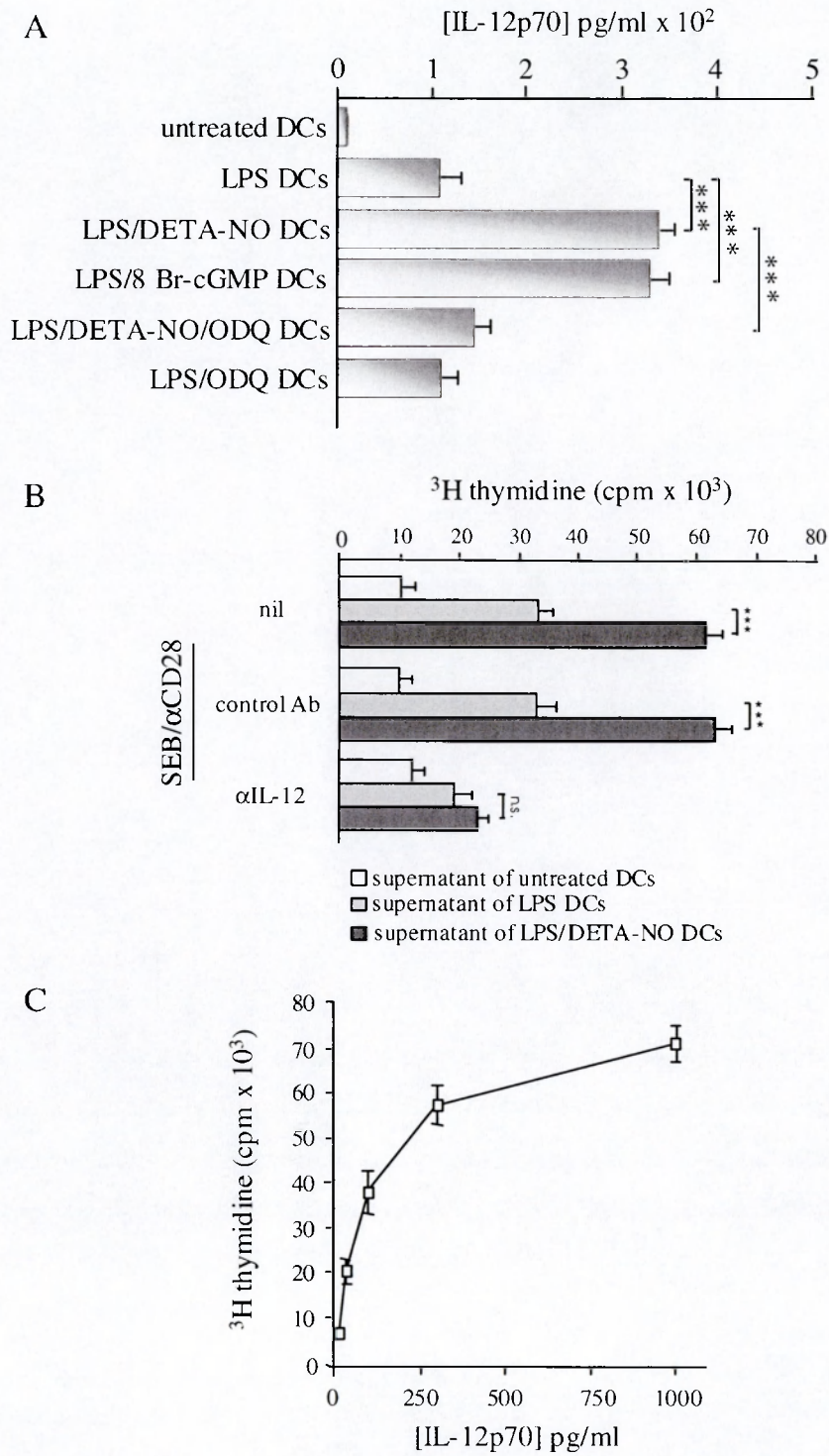


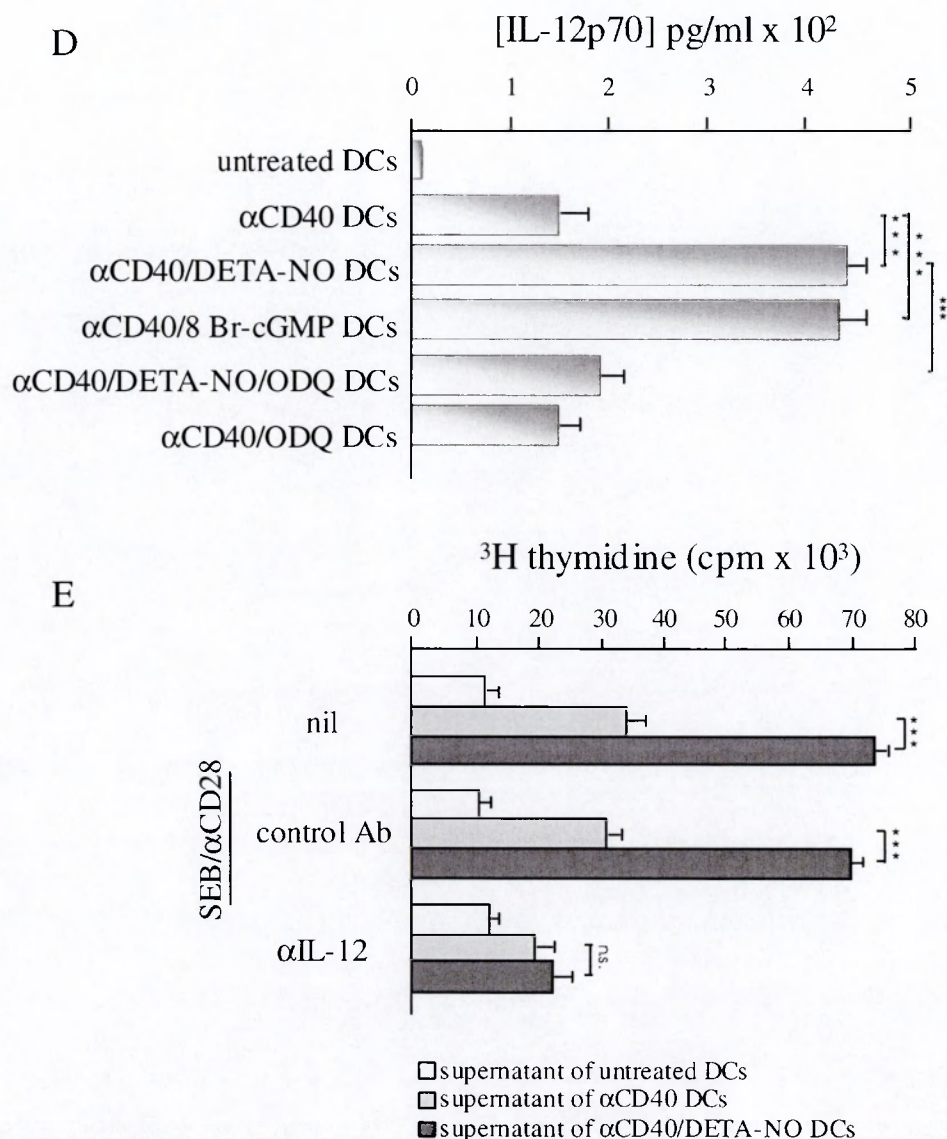
Immature DCs were treated for 2 days with or without LPS (1 μ g/ml) or an anti-CD40 mAb (10 μ g/ml) in the presence or absence of DETA-NO (50 μ M). Cell preparations were analysed by flow cytometry after staining with FITC-conjugated Abs specific for CD80, CD83, CD86 and MHC class II. Values shown are the relative fluorescence intensities \pm SEM measured for each FITC-conjugated Abs vs. cell autofluorescence ($n = 6$).

Since these effects of NO were similar to those observed in TNF- α -treated DCs, I investigated whether they were due to IL-12 release. As already reported (Cella et al., 1996), treatment of DCs with either LPS or anti-CD40 Ab results in the release of IL-12 amounts greater than those observed after DC treatment with TNF- α . These values were further, significantly increased by the co-incubation of DCs with LPS or anti-CD40 together with DETA-NO (Fig. 25, A and D). All the co-stimulatory effects of NO on the production of IL-12 induced by LPS- and anti-CD40 were also dependent on cGMP generation, since they were mimicked by 8 Br-cGMP and reversed by ODQ (Fig. 25, A and D).

I then investigated the effects on T cell proliferation in the anti-CD28/SEB system of supernatants obtained from 2 day cultures of DCs stimulated with either LPS or anti-CD40 mAb, prepared as described above for the experiments with TNF- α . Both conditions gave rise to T cell proliferation, which was significantly increased when supernatants were obtained from DCs incubated also with DETA-NO (Fig. 25, B and E). Administration of the neutralising anti-IL-12 Ab cocktail reduced T cell proliferation induced by the supernatant from either LPS or anti-CD40-pre-treated DCs, and abolished the further enhancing effect induced by DETA-NO.

Figure 25 Release of IL-12 is crucial to NO-potentialiation of the T lymphocyte activation responses by DCs treated with either LPS or anti-CD40 mAb





Supernatants were collected from DCs pre-treated for 2 days with or without either LPS (1 µg/ml, panels A and B) or an anti-CD40 mAb (10 µg/ml, panels D and E) in the presence or absence of either DETA-NO (50 µM), 8 Br-cGMP (3 mM) or ODQ (3 µM). (A and D) Concentration of IL-12p70 in the various supernatants was evaluated by ELISA. (B and E) T cell proliferation in the presence or absence of the various supernatants, as indicated on the left hand side, was stimulated for 3 days with SEB (0.2 µg/ml)/anti-CD28 mAb, in the absence (nil) or presence of an IL-12 neutralising mAb cocktail (αIL-12), as described in the Methods section. Experiments with an isotype control Ab were carried out in parallel. Asterisks indicate statistical significance, measured as indicated in Materials and methods ($n = 6$). (C) T cell proliferation was stimulated for 3 days with SEB (0.2 µg/ml)/anti-CD28 mAb, in the presence of increasing concentrations of IL-12 (0-1000 pg/ml) ($n = 5$).

To further evaluate the role of released IL-12, I measured the effects of adding increasing concentrations of exogenous IL-12 to T lymphocytes in the anti-CD28/SEB experimental system. As shown in Fig. 25 C, IL-12 increased T cell proliferation in a concentration-dependent manner. Moreover, the effects of exogenous IL-12 appeared in the range of those induced by supernatants containing comparable concentrations of endogenously generated IL-12 (compare panel C in Fig. 25 with panels B and E in the same Fig. and with panel B in Fig. 22).

6 DISCUSSION

During the past twenty years, NO has been recognized as one of the most versatile players active on the immune system. This short-lived messenger is generated during immune responses mainly by iNOS, expressed in phagocytes after their activation by cytokines and bacterial products (Moncada et al., 1995). The effects of NO on the immune system are multiple and complex. NO endows activated macrophages and microglial cells with anti-microbial and cytotoxic activity, influences the function of mast cells, neutrophils, T and NK cells, and regulates the generation of cytokines and chemokines at the site of infection (Bogdan 2001). Targeted deletion of iNOS renders mice more susceptible to infections (Wei et al., 1995). Invading pathogens and/or inflammatory stimuli, e.g. TNF- α , LPS and IL-1B, elicit maturation of DCs; simultaneously, in inflamed peripheral tissues DCs are exposed to a variety of signals, among which is NO (MacMicking et al., 1997; Bogdan 2001). Although DCs are considered to have a central role in the induction of specific immune response, so far there is still little information about how NO can affect human DC function. My PhD project was aimed at investigating the role of NO during DC maturation. This complex process is characterized by profound changes in DCs: downregulation of endocytic ability; increased expression of costimulatory molecules, MHC class I and II; increased efficiency in inducing T cell stimulation. In the first part of my thesis I have concentrated the investigation on the effects of NO on the endocytic activity of DCs exposed to a maturation stimulus, and on the mechanism of NO action in the regulation of this process. I have used immature human monocyte-derived DCs which efficiently endocytose antigens *in vitro* (Sallusto et al., 1994). These cells were exposed to TNF- α

which triggers an *in vitro* maturation process, as confirmed by the concentration-dependent reduction of endocytic activity and by the upregulation of molecules involved in T cell activation (Banchereau et al., 1998; Sallusto et al., 1994). Endocytosis was studied by cell exposure to FITC-dextran. As a source of NO I used both NO donors and the murine microglial N9 clone cells, which expresses iNOS after treatment with INF- γ and polystyrene microspheres. The microglial-DC cocultures offer the opportunity of a continuous release and a physiological source of NO. In immature DCs NO, generated either by activated N9 phagocytes or by two NO donors, DETA-NO and SNAP, did not modify endocytosis *per se*. In the presence of TNF- α , however, NO reversed the inhibitory effect of the cytokine, *i.e.* it maintained the ability of DCs to internalise FITC-dextran. Soluble guanylate cyclase, a heterodimeric enzyme that converts GTP to cGMP, is a well known receptor of NO, and mediates the effects of this gaseous molecule in many physiological systems (Denninger et al., 1999). DETA-NO activates soluble guanylate cyclase in immature DCs, leading to a subsequent increase in the concentration of cGMP. Accumulation of this mediator is prevented when DCs are treated with the NO donor in the presence of the soluble guanylate cyclase inhibitor ODQ. Since NO effect on endocytosis was prevented by ODQ, and mimicked by the membrane permeant analogue of cGMP, 8-Br-cGMP, I conclude that NO acts via a cGMP-dependent mechanism.

Maturation of DCs is triggered by TNF- α via activation of its p55 receptor (Sallusto et al., 1995), which induces in many cell types sphingomyelin breakdown with resulting accumulation of the lipid messenger ceramide (Kolesnick et al., 1998; Perry et al., 1998). Involvement of the latter in the inhibition of endocytosis was documented by previous studies with immature DCs, where ceramide inhibited endocytosis of various

substrates, namely lucifer yellow, horseradish peroxidase and FITC-dextran, suggesting its role as the messenger by which TNF- α downregulates this process (Sallusto et al., 1996). I thus investigated whether the maintenance by NO of the endocytic ability in TNF- α -treated DCs was due to inhibition of ceramide accumulation. Consistent with this possibility, I found that TNF- α induced a time-dependent accumulation of ceramide, which was inhibited by both NO donors and NO released by activated N9 cells. Again cGMP generation accounted for this effect of NO, as demonstrated by experiments with ODQ and 8-Br-cGMP.

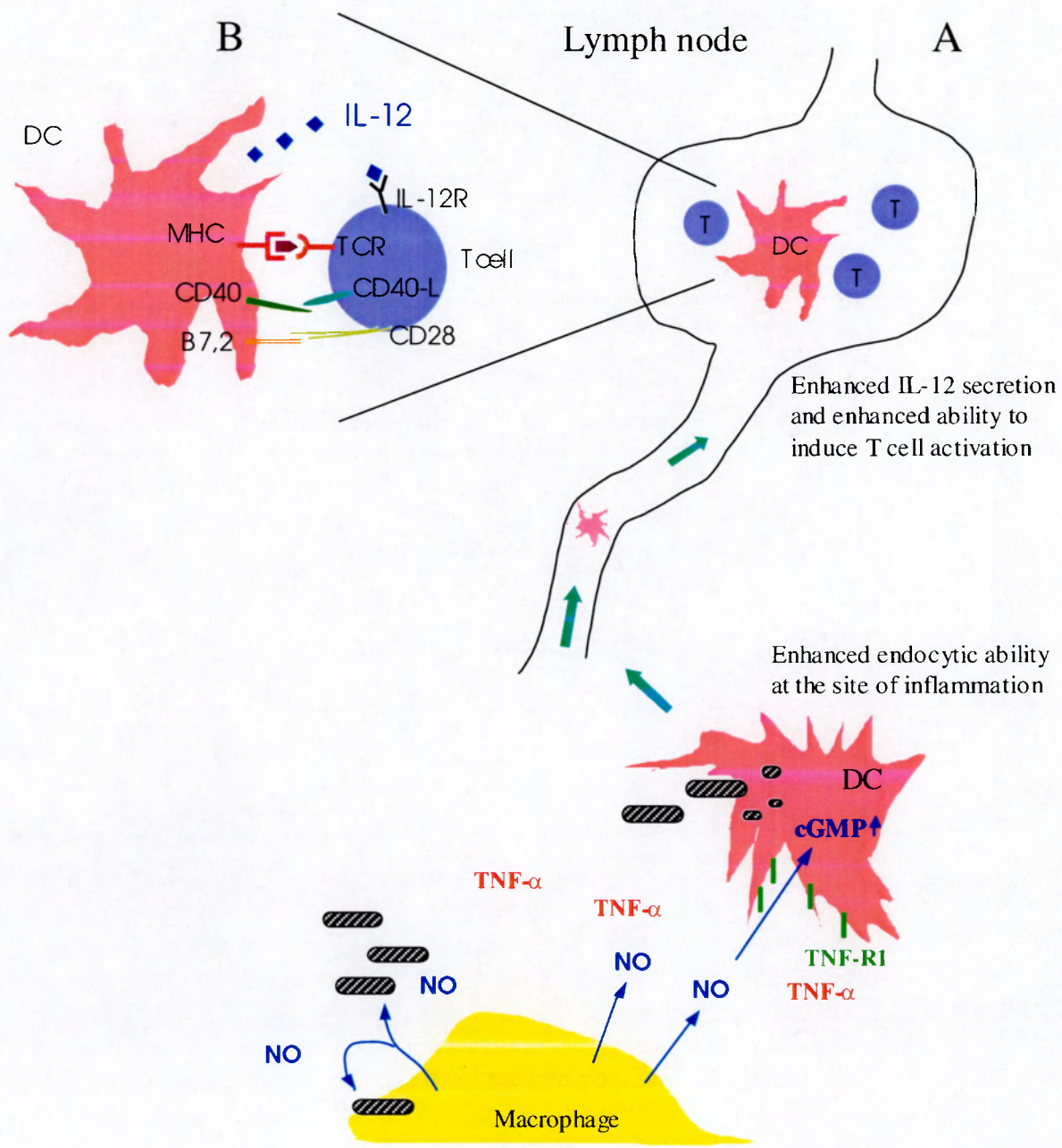
In order to analyse whether inhibition of ceramide accumulation by NO was the only event responsible for its ability to reverse the effect of TNF- α on the endocytic ability of DCs, experiments were carried out with the membrane permeant C₂-ceramide. This lipid inhibited endocytosis of FITC-dextran in a persistent way, however NO and 8-Br-cGMP were still able to reverse this effect. This finding indicates that the action of NO on endocytosis can be explained not only by its cGMP-dependent inhibition of ceramide accumulation but also by additional effects, mediated through cGMP, on the signal transduction pathway activated by TNF- α taking place after the biogenesis of ceramide. The molecular target(s) of this further action by NO/cGMP remain(s) to be established. These results indicate that the inhibition by NO, via cGMP, of the TNF- α -induced downregulation of endocytosis is exerted at multiple levels along the signal transduction cascade triggered by this cytokine. Nitric oxide and cGMP function therefore as wide inhibitors of the action of TNF- α on endocytosis rather than as selective regulators of one single transductional event.

Nitric oxide generation in peripheral tissues occurs as a consequence of various stimuli (MacMicking et al., 1997; Moncada et al., 1995; Liew 1995; MacLean et al.,

1998). The regulation of DC endocytosis by NO through cGMP might enable these cells to prolong their antigen uptake function at the site of inflammation and therefore modify the ensuing immune responses in the lymphoid organs. So far, the role of NO in the maturation process of DCs has been investigated in vitro in the murine system, by measuring antigen presentation as well as the ensuing T cell proliferation after DCs exposure to cytokines, which results in expression of iNOS by DCs (see e.g. Lu et al., 1996). In these systems a reduction in allostimulation was observed at high DC:T cell ratio, and correlated with increasing concentration of NO. It was also demonstrated an induction of apoptosis in DCs by NO (Bonham et al., 1996). These findings suggest an inhibitory role for NO on DC ability to induce an immune response. Regulation by NO of human DC function appears, however, to be different. To my knowledge, iNOS expression and NO generation by these cells had not been reported except in primary biliary cirrhosis and hepatocellular carcinoma (Yamamoto et al., 1998; Ninomiya et al., 1999). Consistent with most previous results, I could detect neither expression of iNOS nor NO generation by human DCs exposed to various combinations of cytokines and LPS. Regulation of human DCs might therefore depend on exogenous NO, generated at the site of infection by macrophages as a result of their activation by cytokine during the inflammatory response (McInnes et al., 1996). Since the functional effects of TNF- α on human DCs appear to be reversible and re-inducible (Nelson et al., 1999), NO might maintain active endocytosis of antigens as long as DCs are confined to the inflammation site, tuning their response to this cytokine and possibly also to other maturative stimuli. After leaving the inflammation site, DCs would no longer be exposed to NO, and could therefore down-regulate their endocytic ability to prevent any interference by irrelevant self-antigens captured during migration to lymphoid organs (Banchereau et al., 1998).

Thus NO, acting on human DCs in a paracrine fashion appears to contribute enhancing the immune responses, whereas dysregulation of its homeostasis, with its generation under severe pathological conditions in DCs (Wang et al., 2001; Yamamoto et al., 1998; Ninomiya et al., 1999), might instead impair the immunological function of these cells. A hypothetical model for this action by NO is in Figure 26.

Figure 26 Proposed model of the effect of NO on DCs during their maturation process



In the second part of my project I have addressed the possibility that NO affects other events involved in the maturation process of human DCs; in particular I have focused my attention on NO modulation of DC ability to induce T cell activation. Recent reports demonstrated that NO, acting in a dose dependent manner, plays an important role in the selective regulation of immune response by modulating T cell functions and differentiation. Low doses of NO can enhance the induction of Th1 cell differentiation by IL-12 and antigens, whereas high doses of NO inhibit the induction of the same cells (Niedbala et al., 1999). Cohen et al. have investigated the role of NO in the human lymphocyte responses to bacterial superantigen. They demonstrated that NO is an essential requirement for lymphocytes proliferation and lymphokine release induced by superantigen stimulation of human mononuclear cells. In addition, they found a marked difference in nitrite production between superantigen-stimulated murine splenocytes and human mononuclear cells. While cultured murine splenocytes stimulated with mitogens can produce nitrite concentrations of 20 to 100 μ M, nitrite production by superantigen-stimulated human mononuclear cells remains at the lower limit of detection. Nitric oxide concentration could explain the discrepancy with previous findings in the mouse, where NO was shown to act as an inhibitor of superantigen induced activation (Sriskandan et al., 1996). So far, even if NO appears to be a modulator of T cell specific immune responses there are no clear studies describing if NO can influence T cell responses by acting on DC function. In view of my findings that, at variance with their murine counterparts, DCs from healthy human donors are unable to express iNOS, I designed the experimental protocol to mimick the in vivo situation of human DCs exposed only to NO generated at the site of inflammation by neighbouring, NOS-competent cells. Accordingly, I exposed DCs to NO only during

their maturation, which was triggered by three different stimuli, TNF- α , LPS and an activating, anti-CD40 mAb. The NO donor and the maturation stimuli were then removed before initiating the co-culture of DCs with T lymphocytes. This approach has allowed not only to mimic the effects of a temporary exposure of human DCs to the gaseous messenger, but also to dissect the effects of NO which are accounted for by a direct action on DCs. Under these conditions I found that NO, while ineffective when administered alone, synergises with each of the three maturation stimuli to trigger a modified DC maturation programme, which results in an enhanced DC ability to stimulate T lymphocyte proliferation. This effect of NO has three characteristics. It is independent of actions on antigen uptake/processing, since it was observed in MLRs and in autologous reactions occurring in the presence of SEB, anti-CD3 mAb or PPD. Nitric oxide effect was observed also with the supernatant of DCs pretreated with the different maturation stimuli in the α CD3/ α CD28 T cell activation system, which is independent of the presence of DCs. In addition, the enhanced DC ability of stimulating T lymphocyte proliferation was found to persist after removal of NO. This latter result suggests that priming with NO in peripheral tissues may be sufficient to exert a licensing effect on antigen presentation in lymphoid organs. Furthermore, the effect of NO appears independent of the stimulus that triggers DC maturation since it was equally observed with LPS, TNF α or the anti-CD40 mAb. This, together with the observation that the increased ability by NO to activate T cells was only observed when both NO and each of the above stimuli were co-administered to DCs, indicates that the gas acts through regulation of specific transductional events activated by these maturation stimuli. Paracrine NO, therefore, appears to function as a co-signal influencing the cross-talk between DCs and T cells (Fig 26).

I also investigated the possible mechanism and signaling events through which NO exerts its action on maturing DCs. Similarly to what has already been described for the regulation of DC endocytosis, I found that the effect of NO depends on the activation of guanylate cyclase and generation of cGMP, as demonstrated by experiments with 8-Br-cGMP and ODC. As far as the signaling events regulated by NO/cGMP I did not observe any modification in the maturation-regulated expression of MHCs and/or major co-stimulatory molecules. However, I found that NO modified the ability of DCs to release molecules to the extracellular milieu. This was clearly shown by experiments carried out using supernatants from DCs treated with the maturation stimuli TNF α , LPS and anti-CD40 mAbs. When DCs were treated with these stimuli in the presence of NO, the supernatants showed enhanced ability to induce proliferation of T lymphocytes activated with the antiCD3/antiCD28 mAbs or SEB/antiCD28 mAb in the absence of APC. I then investigated the nature of released substances involved in mediating the effects of NO on the DC-T cell system. With all three maturation stimuli tested I identified the increased release of IL-12 by DCs as the relevant event occurring in response to the cGMP-dependent effect of NO. I studied the involvement of IL-12 by two experimental approaches. In the first, IL-12 neutralising Abs, when added to the supernatants from the various DC cultures, were found to inhibit the enhancing effect of NO on T cell proliferation. In the second approach, IL-12 added to T lymphocytes in the anti-CD28/SEB system was shown to trigger T cell proliferation in a concentration-dependent fashion, as already reported in a similar system (Kubin et al., 1994). This effect was in the range of that induced by supernatants containing comparable concentrations of IL-12 endogenously produced by DCs exposed to NO and either LPS, TNF α or the anti-CD40 mAb. From these two experimental approaches I concluded that

increased release of IL-12 explains the observed effect of NO/cGMP-treated DCs on T lymphocyte proliferation.

Activation of guanylate cyclase and generation of cGMP mediate many effects of NO in a variety of cells, including signalling events activated by TNF α and LPS (Bulotta et al., 2001; Browning et al., 1999; Harbrecht et al., 1995; De Nadai et al., 2000). Despite the relevant role played by NO/cGMP in immune responses, however, evidence about cGMP regulating maturation signal transduction pathways in DCs had not been reported. The findings I describe here, showing a cGMP-dependent inhibition by NO of the TNF- α -triggered ceramide generation with maintenance of endocytosis, together with the identification of IL-12 as a target of the NO/cGMP action in three distinct signal transduction pathways, may provide a clue for elucidating the molecular events regulated by the cyclic nucleotide during DC maturation.

Generation of IL-12 by DCs is known to be a tightly regulated event (Ma et al., 2001). Production of this cytokine can be elicited by most pathogens and increased further by activated CD40 ligand-positive T cells (Schulz et al., 2000). This soluble mediator is able to activate NK and T cells for the induction of IFN- γ production and cytolytic activity. In part through its induction of IFN- γ production, IL-12 enhances the phagocytic and bactericidal potential of phagocytes and their ability to release proinflammatory cytokines such as IL-1 β , IL-6, TNF- α and IL-12 itself. In addition to its role in mobilizing the first line of defense, IL-12 produced during the early phases of an infection is also fundamental for the ensuing antigen-specific immune response by promoting the differentiation and function of T helper 1 (Th1) cells that support cell-mediated immunity, cytotoxic T cell generation and activation of phagocytic cells. Particularly in the presence of costimulation, IL-12 is also a potent mitogen for T and

NK cells (Ma et al., 2001). Although IL-12 secretion occurs commonly during DC maturation, there are specific situation in which this does not occur. For example, DCs matured in the presence of cholera toxin or CD95 ligand fail to secrete IL-12 (Rescigno et al., 2000). Similarly, pro-inflammatory cytokines such as IL-1 β and TNF- α , appear poor activators of IL-12 secretion, as also shown by my results (Rescigno et al., 2000). The results I have obtained with TNF- α , however, provide evidence that this phenotype can be reversed in the presence of NO.

LPS, TNF- α and CD40 ligand are capable of upregulating iNOS in immune cells competent to express the enzyme, with subsequent, sustained generation of NO (Bogdan 2001). Nitric oxide, therefore, might be part of a feed-forward loop aimed at amplifying immune responses through an increased antigen presenting function of DCs. This novel function of NO might contribute to explain the mechanisms by which cooperation among IL-12, NO and other Th1-type cytokines play an important role in healing of in vivo infections (Das et al., 2001; Perez-Santos et al., 2001; Uzonna et al., 2001; Wilhelm et al., 2001).

6.1 Concluding remarks and future directions

Protective immunity results from the complementary contributions of two fundamental systems: innate and adaptive immune system. The interplay between these systems is critical for the successful detection and elimination of infectious pathogens. Macrophages, NK cells and DCs are sentinels which control pathogen entry and coordinate early defence by innate, and later by the acquired, immune system. At the interface between the innate and the adaptive immune system lies NO, which has been demonstrated to be produced in birds, molluscs, horseshoe crabs, insects, protozoa, and slime molds (MacMicking et al., 1997). Its role in immunity is highly conserved over the course of evolution, supporting the hypothesis of the common evolutionary origin of the natural immune and inflammatory response (MacMicking et al., 1997; Bogdan et al., 2000). Indeed NO is not only a necessary component of non-specific, innate defence mechanisms against pathogens but also modulates several functions of immune competent cells involved in the adaptive responses during inflammation. Thus, NO appears to play both regulatory and effector roles. The work here presented provides first insights about how NO exerts a modulatory role on DCs, both at the level of intracellular signalling events and in terms of final effect on DC function. These findings describe two novel NO functions that link innate and adaptive immunity: NO as a modulator of DC endocytosis, inducing increased uptake of putative antigens, and at the same time as an enhancer of DC ability to activate specific T cell responses. So far, various mechanisms by which DCs were able to adapt their properties as a function of their localization and intercellular interactions have been demonstrated. Our results introducing NO as a novel regulator of DCs suggest new interesting perspectives, because of peculiar aspects of its generations and unique physico-chemical properties.

Our results may open the way to the identification of others intracellular targets, which can mediate NO effects in DCs and to the fine characterization of signalling events modulated by NO during DC maturation induced by various stimuli. Another interesting aspect is the identification of DC plasma-membrane molecules regulated by NO and involved in DC-T cells interactions. It will be also important to investigate whether NO regulates another key event in maturation of DCs, i.e. their migration to lymphoid organs. A fascinating hypothesis is that NO regulates expression of chemokines receptors and adhesion molecules which are known to be modulated during maturation stimuli in order to allow egress from the site of inflammation and migration to lymphonodes (Sallusto et al., 1998).

7 EXPERIMENTAL PROCEDURES

7.1 Materials

The following reagents were purchased as indicated: mouse monoclonal anti-iNOS from Transduction Laboratories (Lexington, KY); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Abs) from Santa Cruz Biotechnology (Santa Cruz, CA); fluorescein-iso-thiocyanate (FITC)-labelled mouse monoclonal Abs anti-human CD1a, MHC class I and class II, CD80, CD83, CD86, CD40 and CD14 from Caltag (Burlingame, CA); γ 32 P-ATP and the cGMP radioimmunoassay kit from NENTM (Boston, MA); the Enhanced ChemiLuminescence kit from Amersham Corporation (Little Chalfont, UK); recombinant human TNF- α , (Z)-1-[2-(2-aminoethyl)-N-(2ammonioethyl) amino]diazen-1-ium-1,2 diolate (DETA-NO) and H-[1,2,4]oxadiazolo[4,3- α]quinoxalin-1-one (ODQ) from Alexis Italia (Florence, Italy); diacylglycerol kinase from BIOMOL (Hamburg, Germany); C₂ ceramide, aminoguanidine and S-nitroso-acetylpenicillamine (SNAP) from Calbiochem (Bad Soden, Germany); recombinant human interleukin-4 (IL-4) from Strathmann Biotech GMBH (Hannover, Germany); recombinant human Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) from Mielogen-Schering Plough (Milan, Italy); recombinant mouse and human interferon- γ (IFN γ) from Genzyme (Cambridge, MA); FITC-dextran (Mr = 40,000) from Sigma (Milan, Italy); Ficoll from BioChrom (Berlin, Germany); Percoll from Pharmacia Biotech (Milan, Italy); polybed polystyrene microspheres from Polyscience Inc. (Milan, Italy). The reagents for tissue culture were from GIBCO (Basel, Switzerland) except for foetal calf serum (clone III), obtained from Hyclone-Celbio, (Milan, Italy). The N9 murine microglial cells were kindly provided by

Paola Ricciardi-Castagnoli (Milan, Italy). The mAb anti-human CD28 was from Becton Dickinson (San José, CA); methyl-³H thymidine from Amersham Pharmacia Biotech (Buckinghamshire England); *Mycobacterium tuberculosis*-derived tuberculin purified protein derivative (PPD) from Statens Serum Institut (Copenhagen, Denmark); magnetic Dynabeads M-450 goat anti-mouse IgG from Dynal (Oslo, Norway); the anti-human IL-12 neutralising mAbs (clones 17F7 and 20C2) were kindly provided by Lars Rogge (Roche Milano Ricerche, Milan, Italy). The anti-CD40 mAb, purified from the 626.1 hybridoma, was kindly provided by D. Vercelli (Tucson, AZ, USA). The mAb anti-human CD3 was purified from the hybridoma (OKT3) obtained from American Type Culture Collection (Rockville, MD). *Staphylococcus aureus* Cowen strain B (SEB) and all of the other reagents were from Sigma (Milan, Italy).

The conditions of use of DETA-NO, 8 Br-cGMP and ODQ were selected on the basis of previous experiments in monocytes and macrophages. In particular, at the concentrations employed in this study, these compounds appear to be not toxic while still able to trigger biological effects (Clementi et al., 1998; Orsi et al., 2000).

All solutions were prepared endotoxin-free. Endotoxin contamination in all drug solutions was routinely assessed by the Lymulus amoebocyte gelification test, carried out according to the manufacturer's instructions (PBI, Milan, Italy), before administration to DCs. All reagents, including the medium, scored negative.

7.2 Preparation of immature and mature dendritic cells

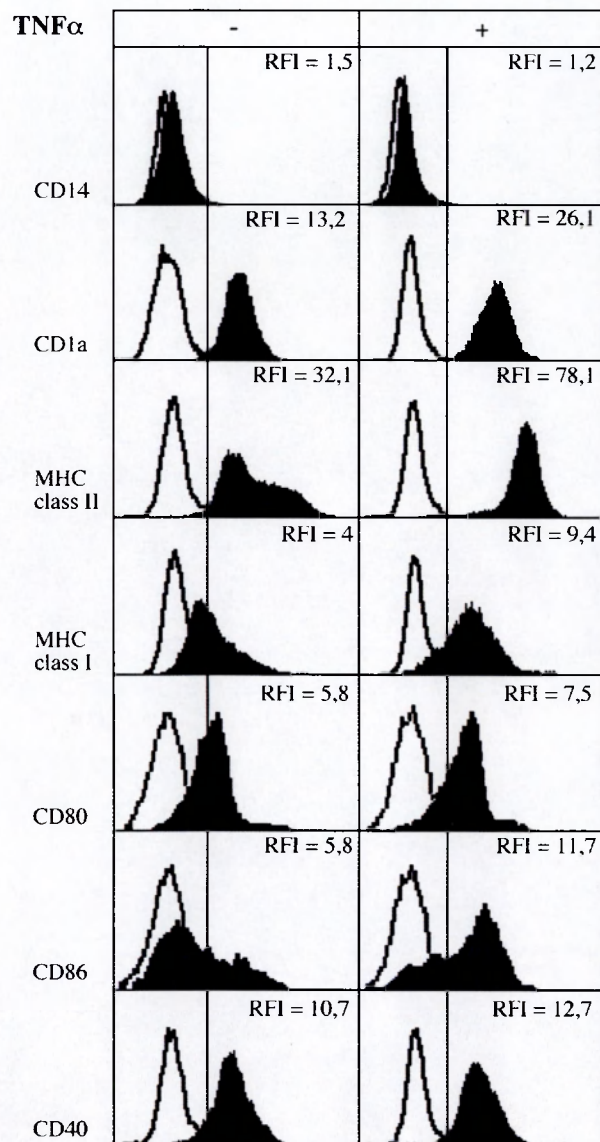
Peripheral blood mononuclear cells from healthy blood donors were obtained either from buffy coats (kindly provided by the Blood Transfusion Department of our Institution) or heparinised blood drawn by venipuncture. Standard gradient separation

procedures on Fycoll-Paque were used (Sallusto et al., 1994). PBMC were resuspended in RPMI-1640 containing 10 % FCS, 2 mM glutamine 100 U/ml penicillin 100 µg/ml streptomycin, from hereon referred to as “complete medium”, and allowed to adhere on 6-well plates (Costar, Cambridge, MA) for 1 h at 37°C in a humidified 5% CO₂ atmosphere. Non-adherent cells were discarded. In order to derive immature DCs, PBMC were then cultured for 7 days in complete medium, in the presence of human GM-CSF (50 ng/ml) and IL-4 (1000 U/ml). Cells were refed with fresh complete medium containing GM-CSF and IL-4 on days 2, 4, and 6. Depletion of residual T lymphocytes from immature DCs was routinely carried out by incubating the cell preparations with the anti-CD3 mAb (1 µg/10⁶ cells) and goat anti-mouse IgG-coated Dynabeads M-450. Anti-CD3 mAb-bound T cells were removed with a magnet according to manufacturer’s instructions. Purified DC preparations were routinely checked for contaminating lymphocytes by flow cytometry using a fluorescence-activated cell sorter (FACScan plus, Becton Dickinson, Sunnyvale, CA). Residual lymphocytes accounted for less than 1 % of total cells (not shown). Mature DCs were obtained from immature DCs by a 48 h incubation in complete medium in the presence of human TNF-α (0.2-200 ng/ml), anti-CD40 mAb (10 µg/ml) and LPS (1 µg/ml). Similar conditions were used in the experiments in which C₂-ceramide (80 µM) was used. In the experiments in which DCs were incubated with TNF-α, LPS and anti-CD40 mAb maturation was routinely assessed by flow cytometry, measuring the exposure on the plasma membrane of specific antigens known to be expressed by immature or mature DCs, namely CD1a, a marker of human myeloid DCs; MHC class I and class II molecules; CD80 and CD86, involved in T cell co-stimulation (Sallusto et al., 1994) (Chen et al., 1998). Expression of these antigens was analysed after staining with

appropriate FITC-labelled Abs as described later. Figure 27 shows results from a typical analysis: GM-CSF, IL-4- treated (immature) DCs express CD1a, MHC class I and class II, CD80, CD86 and CD40. TNF- α -treated (mature) DCs show a significant upregulation of MHC class I and class II, CD1a, CD80, CD86 and CD40. Expression of the macrophage marker CD14 was never observed. Viability and apoptosis in the various samples was assessed at different times by propidium iodide/annexin V staining exactly as described (Rovere et al., 1996).

When the 48 h incubation with either TNF- α or C₂-ceramide was carried out in the presence of DETA-NO (100 μ M), SNAP (200 μ M), 8-Br-cGMP (3 mM) or ODQ (3 μ M), the compounds were added in various combinations 10 min before and were maintained throughout the incubation time. Control experiments in which DCs were incubated with the various compounds in the absence of TNF- α and C₂-ceramide were carried out in parallel. In the experiments in which cells were exposed to DETA-NO and SNAP, they were dissolved immediately before addition to the cells. SNAP and DETA-NO prepared 7 days before the experiments were used in control experiments. Under these conditions they do not release any NO, as measured using a NO detecting electrode with a sensitivity of 1 nM (Mark-2 ISO NO, World Precision Instruments, Sarasota, FL). Before the co-culture with lymphocytes, DCs were washed twice in complete medium, to remove all the various compounds, and then irradiated (2500 rad). Removal of the NO donor was routinely checked by verifying absence of NO generation in washed DC suspensions using a NO electrode (Clementi et al., 1998).

Figure 27 Phenotypic characterisation of human DCs exposed to TNF- α .



Human monocytes were treated with GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) for 7 days to obtain immature DCs. These cells were then incubated with or without TNF- α (200 ng/ml) for further 48 h (right and left columns, respectively). Cell preparations were stained with FITC-conjugated Abs for surface antigens, as specified on the left hand side, and analysed by flow cytometry as described in the Experimental procedures (filled histograms). Their relative fluorescence intensity (RFI) was calculated vs negative controls (open histograms). The results shown are from one representative experiment.

7.3 Co-culture of dendritic cells and nitric oxide-generating N9 cells

Exposure of DCs to a continuous flux of NO was achieved *in vitro* using the scavenger murine microglial brain N9 clone cells derived from embryonic mouse, which express iNOS upon activation (Corradin et al., 1993). N9 cells were cultured in Iscove's modified DMEM containing 10% foetal calf serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine. Induction of iNOS by these cells requires IFN-γ together with a second signal (Corradin et al., 1993). To induce iNOS expression, N9 cells (2×10^5 cells/ml) were incubated for 24 h in the presence of polybed polystyrene microspheres (microsphere/cell ratio = 5) and mouse IFN-γ (10 U/ml), with or without the NOS inhibitors aminoguanidine (1 mM) and N^ω-nitro-L-arginine methyl ester (L-NAME; 1 mM). Nitric oxide production by N9 cells was measured by determining the nitrite accumulation in the culture medium using the Griess reaction (Green et al., 1982). Standard curves with increasing concentrations of sodium nitrite were run in parallel. The co-culture of DCs and N9 cells was carried out using a double chamber system (Costar, Cambridge, MA). A semi-permeable polycarbonate membrane with a cut-off of 0.4 µm separated the lower chamber, containing immature DCs, from the upper chamber. Dendritic cells were incubated for 48 h in the lower chamber at a density of 1×10^6 cells/well either in the presence or absence of human TNF-α (200 ng/ml). Activated, iNOS-expressing N9 cells were seeded in the upper chamber at a density of 2.5×10^5 cells/well at the beginning of the incubation with TNF-α. Dendritic cells were then collected and endocytosis of FITC-dextran was measured as described below.

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7.4 Flow cytometric analysis

To assess DC phenotype after the various stimuli, cells were washed with PBS and then 5×10^5 cells were incubated for 30 min at 4°C in PBS containing 1 % FCS with FITC-conjugated mAb specific to MHC class I, MHC class II, CD1a, CD14, CD40, CD80, CD83 or CD86. Cells were then washed and resuspended in 300 ul of cold PBS containing 1 % FCS. Expression of the various molecules was evaluated by flow cytometry using a fluorescence-activated cell sorter (FACStar Plus, Becton Dickinson, Sunnyvale, CA) . Residual lymphocytes and cellular debris were eliminated from the analysis using a gate on forward and side scatter. A minimum of 10^4 DCs were analyzed for each sample. Results were processed with a Cell Quest software (Becton Dickinson).

7.5 Analysis of endocytosis of FITC-dextran

Dendritic cells, exposed to the various treatments described in the paragraphs above, were washed and resuspended in complete medium. 2×10^5 cell/samples were incubated in 200 ul medium at either 37 or 4°C with FITC-dextran (1 mg/ml). Uptake of the fluorescent dye was stopped at the indicated time-points by the addition of ice-cold phosphate buffered saline containing 1% foetal calf serum. Samples were then washed three times in the same buffer at 4 °C and analysed by flow cytometry using propidium iodide to exclude dead cells as described (Lutz et al., 1997). FITC-dextran, reconstituted in RPMI and stored at 4°C, was centrifuged to remove aggregates before addition to the cells.

7.6 Measurement of cGMP generation

Immature DCs (1×10^6 cells/sample), incubated for 15 min at 37 °C in phosphate buffered saline with the phosphodiesterase inhibitor hydroxy buthyl methyl xanthine (0.6 mM), were incubated for an additional 15 min in the presence or absence of DETA-NO (100 μ M), with or without ODQ (3 μ M). The reaction was terminated by addition of ice-cold trichloroacetic acid (final concentration: 7.5 %). After ether extraction, cGMP levels were measured using a radioimmunoassay kit and normalised on cellular proteins, determined by the bicinchoninic acid assay procedure (BCA protein assay; Pierce, IL).

7.7 Measurement of ceramide concentrations

Immature DCs (1×10^6 cells/sample) were incubated in 80 μ l phosphate buffered saline with human TNF α (200 ng/ml) in the presence or absence of either DETA-NO (100 μ M), SNAP (200 μ M) or 8-Br-cGMP (3 mM), with or without ODQ (3 μ M), then quickly shifted at 37°C. At the time-points indicated, incubation was stopped by the addition of 300 μ l ice-cold CH₃OH/CHCl₃ (2/1, vol/vol). Samples were then supplemented with 100 μ l CHCl₃ and 100 μ l NaCl (1M). The extracted phospholipids were incubated for 1 h at room temperature with 100 μ U diacylglycerol kinase in the presence of 5 mg/ml cardiolipin, 7.5 % glucopyranoside, 1 mM diethylenetriamine pentaacetic acid and 10 μ Ci γ -³²P-ATP (10 mCi/ml) as described (Cifone et al., 1994). Under these conditions, diacylglycerol kinase is not rate limiting and full conversion of ceramide to ceramide phosphate is thus to be expected (Perry et al., 1999). The ceramide phosphates produced were separated by thin layer chromatography (Silca gel 60, Merck, Milan, Italy) using CHCl₃/CH₃OH/CH₃COOH (65/15/5, vol/vol/vol) as

solvent. To determine the concentration of ceramide per sample, known amounts of ceramide standard were processed and loaded in parallel. The relevant spots were identified by autoradiography and their radioactivity estimated by microdensitometry using a Molecular Dynamics Imagequant apparatus (Buckinghamshire, UK).

7.8 Western blotting

Dendritic cells were incubated for 24 h in the culture medium with or without human TNF- α (200 ng/ml), IL-4 (1000 U/ml), GM-CSF (50 ng/ml), IFN γ (100 U/ml) and lipopolysaccharide (LPS, 10 μ g/ml) in various combinations as described in the results. Cells were then collected, washed three times with cold PBS and lysed for 30 min with a buffer containing 150 mM NaCl, 1 mM EDTA, 10% glicerolo, 50 mM HEPES pH 7,5, 15 mM MgCl₂, 1 % Triton X-100, 0.1 mM phenylmethyl sulphonylfluoride, 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. The homogenates were centrifuged at 3000 rpm for 5 min at 4°C and the supernatant was recovered. Protein content in the lysates was assayed by the bicinchoninic acid procedure. After addition of sodium dodecyl sulfate (SDS) and β -mercaptoethanol the samples were boiled for 5 min and 50 μ g of protein/lane were loaded into the slots of 10 % SDS polyacrylamide gels as described (Sciorati et al., 1997). High-efficiency transfer of proteins onto nitrocellulose membranes was obtained at 300 mA for 4 h at 4°C, in a buffer containing 25 mM Tris, 192 mM glycine, 20% CH₃OH, pH 8.3. After transfer, the blots were stained with Ponceau red. For Western blotting, the nitrocellulose membranes were incubated in a blocking buffer (Tris-HCl 50 mM, NaCl 150mM, Tween-20 0,05%, 5% powdered milk) for 1 h at room temperature, then incubated over night at 4° C with the anti-iNOS Ab in the same buffer under continuous agitation. The next day the nitrocellulose sheets

were processed at room temperature: the membranes were washed for 5 min with blocking buffer at least 5 times and incubated for 1 h with HRP-labeled goat anti-mouse Ab, after which the membranes were washed several times in the same buffer. Immunoreactive bands were revealed with Enhanced ChemiLuminescence detection reagent according to the manufacturer's instructions.

7.9 Lymphocytes preparations

T cell enriched preparations were obtained as follows: PBMC (5×10^6), were obtained from PPD positive or negative donors or from buffy coats (kindly provided by the Blood Transfusion Department of our Institution) with a standard gradient separation procedures on Fycoll-Paque as described before. PBMC were washed in RPMI 1640 medium and incubated in complete medium containing 0.5% carbonyl iron for 60 min at 37°C on a rotating wheel. Phagocytic cells engulfed of carbonyl iron were removed with a magnet as described (Burastero et al., 1990). T cell enriched population was separated from residual carbonyl iron with a density Fycoll-Paque gradient and then washed twice in RPMI 1640 medium. With these procedures, residual monocytes accounted for less than 1 % of the cell preparations, as checked with anti-CD14 mAb by flow cytometry (not shown).

7.10 Lymphocyte proliferation assays

For MLRs DCs, pre-treated as described above, were co-cultured at different ratios to 96 well flat bottom plates together with heterologous lymphocytes, which were maintained at the constant concentration of 10^5 cells/well. Dendritic cells to lymphocytes ratios ranged from 1:1000 to 1:5. For antigen-driven proliferation assays

DCs (2×10^4 cells/well) were incubated with autologous lymphocytes (10^5 cells/well) in the presence of 1 $\mu\text{g/ml}$ PPD, 0.2 $\mu\text{g/ml}$ SEB, or 1 $\mu\text{g}/10^3$ cells anti-CD3 mAb. In some experiments, lymphocytes were stimulated either with SEB (0.2 $\mu\text{g/ml}$) on plates pre-coated by a 3 h incubation (37°C) with anti-CD28 mAb (5 $\mu\text{g/ml}$), or on plates pre-coated with both the anti-CD28 mAb and anti-CD3 mAb (1 $\mu\text{g/ml}$) in the presence of a DC-conditioned culture medium (1:1). This DC-conditioned medium was freshly prepared the day of the experiment by collecting supernatants conditioned for 48 h by the various DC preparations described above. The supernatant was filtered through a 0.2 μm Millipore filter before use. T cell proliferation was evaluated by measuring incorporation of methyl- ^3H thymidine (0.8 $\mu\text{Ci/well}$), which was added to the co-cultures during the last 6 h. To this end cells were harvested and radioactivity measured using a Wallac LKB β -counter. The results are expressed as the mean of triplicate cultures.

7.11 Cytokine assays

T cells were co-cultured with DCs (10^5 T cells plus 2×10^4 DCs), pre-treated as described above, on 96-well plates in 200 μl medium. IL-2 and IFN- γ concentrations were determined in the medium 48 h after stimulation using commercially available ELISA kits for human IFN- γ and IL-2 (R&D Systems, Space Import Export, Milan, Italy). IL-12p70 was measured in supernatants conditioned for 18 h by DCs pre-treated with or without TNF- α in the presence or absence of DETA-NO using the Quantikine High Sensitivity kit (detection limit 0.5 pg/ml) following the manufacturer's instructions (R&D Systems).

7.12 Measurement of nitric oxide accumulation

Nitric oxide production was measured by determining the nitrite accumulation from the culture medium of cells using the Griess reaction (Green et al., 1982). Standard curves with increasing concentrations of sodium nitrite were run in parallel.

7.13 Statistical analysis

The results are expressed as means \pm SEM; n represents the number of individual experiments. Statistical analysis was performed using the Student's t test for unpaired variables (two-tailed). The marks **, and *** in the figures panels refer to statistical probabilities (P) of < 0.01 and < 0.001 , respectively, measured in the various experimental conditions as detailed in the legends to figures.

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10 LIST OF ABBREVIATIONS

APC	Antigen-presenting cell
CAT	Cationic amino acid transporter protein
CLA	Cutaneous lymphocyte-associated antigen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DC	Dendritic cell
DETA-NO	(Z)-1-[2-(2-aminoethyl)-N-(2ammonioethyl) amino]diazen-1-ium-1,2 diolate
EAE	Experimental allergic encephalomyelitis
ELC	EBL1 ligand chemokine
FAD	Flavin adenine dinucleotide
FADD	Fas-associated death domain
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FMN	Flavin adenine mononucleotide
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HMGB1	High mobility group 1
Hsp	Heat shock protein
ICD	Intracellular domain
IFN γ	Interferon- γ
IL	Interleukin
ILT	Immunoglobulin-like transcript
ImDC	Immature Dendritic cell
IRAK	IL-1R-associated kinase
ITAM	Immunoreceptor tyrosine-containing activation motif
LAK	lymphokine-activated killing
L-NAME	N ^ω -nitro-L-arginine methyl ester
L-NMMA	L-N ^G -Monomethylarginine, Acetate Salt
LPS	Lipopolysaccharide
MDR-1	MDR-1-type p-glycoprotein

MHC	Major histocompatibility complex
MLR	Mixed lymphocytes reaction
MMR	Macrophage-mannose receptor
NCR	Natural cytotoxicity receptors
NIK	NF- κ B-inducing kinase
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
ODQ	H-[1,2,4]oxadiazolo[4,3- α]quinoxalin-1-one
PBMC	Peripheral blood mononuclear cell
PPD	Tuberculin purified protein derivative
pre-DC	Dendritic cells precursor
RFI	Relative fluorescence intensity
RIP	Receptor-interacting protein
SEB	<i>Staphylococcus aureus</i> Cowen strain B
SLC	Secondary-lymphoid-tissue chemokine
SNAP	S-nitroso-acetylpenicillamine
SODD	Inhibitory protein silencer of death domains
SRs	Scavenger receptors
TCR	T cell receptor
TGF- β	Transforming growth factor-beta
Th1	T helper lymphocyte
THB	Tetrahydrobiopterin
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
TRADD	TNF receptor-associated death domain
TRAF	TNF-R-associated factor

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